Developing a Biological Caries Model & Studying Fluoride in Caries Control

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Abstract

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This thesis examines the development of a novel *in vitro* biological caries model and its suitability in testing the efficacy of anti-caries approaches. Dental caries remains a public health concern worldwide; with extensive treatment costs and impacts on quality of life. Ineffective removal of all dental plaque from tooth surfaces after brushing, the insufficient delivery of anti-caries therapies; along with continuing shifts towards high frequency, sucrose-rich food consumption, expedites the caries disease process. It is, therefore, important to explore caries risk and development at these sites, particularly when representatively assessing the efficacy of a test agent in preventing caries. This caries model enabled the study of the anti-caries effects of fluoride to assess its efficacy in conditions simulating the modern diet. The current methodology employed the Constant Depth Film Fermenter (CDFF) to investigate the caries disease process in response to fluoride delivered continuously; twice and thrice daily; and at different concentrations. The approach is the first in CDFF research modelling caries inclusive of a biologically relevant microcosm biofilm in addition to enamel demineralisation. Specific members of multispecies biofilm were selectively enumerated using traditional microbiological culture techniques whilst caries was simultaneously quantified with Transverse Microradiography (TMR), Quantitative Light-Induced Fluorescence (QLF), and Non-Contact Surface Profilometry (NCSP). The fluorescence of biofilm illuminated by QLF was also investigated. Results indicated that quantities of total or specific members of the microbial community are not direct indicators of caries risk and turning focus towards the metabolism of oral biofilm bacteria, and how it may be affected, is vital in caries research. TMR and QLF agree when quantifying caries whilst NCSP shows promise in studying surface changes. At 0.05 ppm, fluoride was unable to exert a significant anti-caries effect despite being continuously present during and between sucrose exposures. Laminated lesions confirmed the importance of maintaining elevated levels of fluoride in the oral environment throughout the day. At higher concentrations (1,450 and 228 ppm fluoride) the anti-caries efficacy of fluoride when supplied in frequent applications throughout the day was confirmed. A third application of fluoride did not appear to additively benefit enamel, since all strategies were effective after 10 days regardless of frequency. Nonetheless the increased plaque fluoride reservoir and subtle antimicrobial effects than in twice daily pulsed biofilm, mean the benefit of a third application is likely more discernible in the long term. Scanning Electron Microscopy (SEM) and Energy-Dispersive X-ray Spectroscopy (EDX) elucidated significant calcium fluoride deposits of enamel surfaces beneath biofilm exposed to 1,450 ppm fluoride continuously. In conclusion, the CDFF can produce multispecies biofilm under conditions similar to those of the oral milieu and investigate its cariogenicity in response to diet and experimental anti-caries agents. The model could be examined using an array of techniques to obtain information about aspects of the biofilm, the substratum, and to validate upcoming methods in an orally representative environment. In this regard, the current study contributes not only to enamel caries research but to biofilm research in general by minimising variation.

"We are what we repeatedly do. Excellence, then, is not an act, but a habit"

Aristotle

Declaration

This thesis is the result of my own work. The material contained within has not been presented, nor is it currently being presented, either wholly or in part for any other degree or qualification.

The research work was undertaken at the School of Dentistry, Department of Health Services Research, University of Liverpool.

Khush Bakht

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List of Abbreviations

APF	acidulated phosphate fluoride
AHLs	acylated homoserine lactones
ATP / ATPase	adenosine triphosphate / adenosine triphosphatase
ANCOVA	analysis of co-variance
ANOVA	analysis of variance
ABC	ATP-binding cassette
Av	average / statistical mean
BC	Before Christ
CPP-AC(F)P	casein phosphopeptide – amorphous calcium (fluoride) phosphate
ΔF	change in fluorescence
CSP	competence signalling peptide
CLSM	Confocal laser scanning microscopy
CDFF	Constant Depth Film Fermenter
DMFT	Decayed, Missing, Filled teeth
df	degrees of freedom
DNA	deoxyribonucleic acid
EDJ	enamel-dentine junction
EDX	energy dispersive X-ray spectroscopy
EI, EII	enzyme I, enzyme II of PTS
Eqn.	equation
EPS	extracellular polymer substance / Extracellular polysaccharide
FAp	fluorapatite
F^-	fluoride ion
FTFs/Ftf	fructosyltransferases / fructosyltransferase enzyme
F	F-statistic = (*df* factor,*df* error)
GPH	galactose-pentose hexuronide
GTFs/Gtf	glucosyltranferases / glucosyltransferase enzyme
g	gram
HPr	histidine protein

HA	hydroxyapatite
IPS	intracellular polysaccharide
IAP	ion activity product
kV	kilovolt
LED	Light emitting diode
MALDI-TOF MS	Matrix Assisted Laser Desorption/Ionisation: Time-of-Flight Mass Spectrometry
µg	microgram
µL	microlitre
µm	micrometre
µM	micromolar
mA	milliampere
mg	milligram
mL	millilitre
mm	millimetre
mM	millimolar
ΔZ	mineral loss
min	minutes
M	molar
MSM	multiple sugar metabolism
MSI	multi-spectral imaging
MS	mutans streptococci
nm	nanometre
NHS	National Health Service
NCSP	Non-contact Surface Profilometry
ppm	parts per million
r	Pearson's product moment coefficient
PBS	phosphate buffered saline
PEP	phosphoenolpyruvate
PG	phosphoglyeric acid

PTS	phosphotransferase system
PTFE	polytetrafluoroethylene
r^2	proportion of total variance explained in linear regression model
R^2	proportion of total variance explained in multiple regression model
PMF	proton motive force
p	*p*-value, probability that null hypothesis is true
QLF	Quantitative Light-induced Fluorescence
(q)PCR	quantitative polymerase chain reaction
n	sample size
SEM	Scanning Electron Microscopy
KSP	solubility product
SD	standard deviation
SPSS	Statistical Package for the Social Sciences
SSIII	Type III sum of squares
TMR	Transverse Microradiography
UV	ultra-violet
w/v	weight/volume
WHO	World Health Organisation

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Hope C.K., Bakht K., Burnside G., Aitken G.C., Burnett G., de Josselin de Jong E. and Higham S.M. "Reducing the variability between constant-depth film fermenter experiments when modelling oral biofilm" Journal of Applied Microbiology, September 2012, 113(3):601-608. doi:10.1111/j.1365-2672.2012.05368.x

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PRESENTATIONS WITHOUT PROCEEDINGS

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Bakht K., Higham S.M., de Josselin de Jong E., Martin G.C., Burnett G. and Hope C.K. "Development of a biologically relevant caries model" Weybridge Innovation Centre Science Symposium, GlaxoSmithKline, Weybridge, UK, 28 – 29 April 2010.

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Bakht K., Higham S.M., de Josselin de Jong E., Martin G.C., Burnett G. and Hope C.K. "Biological caries model & fluoride remineralisation study" Weybridge Innovation Centre Science Symposium, GlaxoSmithKline, Weybridge, UK, 27 – 28 April 2013.

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Chapter 1 Introduction & review of the literature

1.1 INTRODUCTION

Only two years ago, the World Health Organisation (2012) reported that oral disease is the fourth most expensive disease to treat in industrialised countries (WHO Factsheet 318). In spite of great achievements in improving oral health in populations globally, dental caries remains a public health concern; particularly among underprivileged groups in both developed and developing countries. Since, in general, the consumption of excess fermentable carbohydrates remains excessive in industrialised countries it is unlikely that the problem will appease without effective interventions and on-going research to combat the continuing prevalence of caries. Moreover, despite historically less common and less severe caries manifestation in African countries (Petersen, 2003), it is expected that caries prevalence will increase as a result of rises in sugar consumption and the inadequate exposure to fluoride in these regions. Additionally, current worldwide changes in food consumption habits involving a marked shift away from consumption limited to thrice daily but further toward snacking and "grazing" mean dental plaque bacteria are metabolising carbohydrates more frequently throughout a given day. Subsequently, plaque pH falls below critical pH more regularly and the overall risk of substantial demineralisation significantly increases. Historically, the most significant increases in caries prevalence have been associated with dietary changes (Suddick and Harris, 1990); therefore it is acceptable to anticipate that prevalence may once again markedly increase and that caries remains a major health concern; particularly if current acceptable anti-caries therapies cannot keep up with the demands of modern diets. The search for new and improved anti-caries therapies has been a formidable driving force in the development of appropriate models of the dental caries disease

process. Caries is the localised demineralisation of tooth enamel when biofilm bacteria metabolise dietary carbohydrates into acids. It is therefore important to employ a holistic approach in its study whereby the cariogenicity of biofilm alongside the progression of demineralisation in the associated enamel is investigated. Non-biological *in vitro* model systems do not fully represent the mix of organic acids of microbial origin, nor do they account for the challenge faced by therapeutic agents in penetrating biofilm to reach enamel and aid in remineralisation. The constant depth film fermenter (CDFF) is a laboratory device capable of producing biofilm of a defined thickness; it can mimic the oral environment and has proven a useful tool in the field of dental research (Kinniment *et al.*, 1996a, b; Pratten and Ready, 2010; Zaura *et al.*, 2011). Despite this, a review of the literature reveals that few studies exist wherein CDFF technology has been used to test the anti-caries efficacy of fluoride with a microbiological model system. Furthermore, few *in vitro* models incorporate the inspection of complex multi-species plaque alongside the study of changes in the enamel, since the approach of many models was reductionist; developed to study only specific aspects of the disease or involved only a single species of bacteria. Historically, studies of dental plaque have also propelled our understanding of the nature of biofilm. As a good model biofilm, studies of dental plaque and the diseases it mediates, therefore, contribute to a field of utmost importance in modern microbiology.

The aim of this research is to develop a biologically relevant model of dental caries. By investigating its efficacy in modelling the generally well understood anti-caries action of fluoride, the overall objective is to evaluate the potential capabilities of the model in elucidating the effects of lesser known anti-caries approaches. The literature review to follow provides a basis for the theoretical knowledge and experimental design required for formulating studies in an attempt to achieve these

aims. As argued by Boote and Beile (2005), the importance of a sophisticated literature review is the foundation and inspiration for substantial useful research.

Since dental caries is a biofilm mediated disease process, this thesis begins with an overview of biofilm research; strictly focusing on key findings, and to further argue the importance of studying them. Dental plaque as a model biofilm is defined; exploring the specific features of this type of biofilm; including its structure, properties and stages in its formation. It then transitions into an account of dental caries; highlighting its worldwide disease burden and its impact on quality of life. As a great testament to how strides are made in scientific understanding, the history of our perception of caries phenomena is important and fascinating. As in many fields, pursuing deeper, clearer and more accurate knowledge of the disease has generated a number of conflicting views yet the foresight of much of the creative thinking was invaluable. Even very early theories laid seed for worthwhile research and as the query was unravelled aspects of these theories exist in our modern day understanding of the disease. Therefore, this section is presented somewhat chronologically. From here, the characteristics of the microbial aspects of caries are discussed including the changing theories of its role in the aetiology of caries. The cariogenic features of dental plaque bacteria are described, focusing on the most pertinent literature evidencing the way these features participate in the disease process. Our current understanding of the aetiology of caries and the prevailing opinions on the role of microbiology are defined. Perspective is then shifted to focus on an overview of the chemistry of enamel caries. Here evidence for the structure of enamel, its dissolution and its growth is presented. Subsequently, it seems a logical place to introduce the subject of fluoride and its role in the control of caries. Literature for its two pronged anti-caries effects includes evidence of its influence on enamel and on the microbial-biofilm aspects of the disease process. A discussion of the views and evidence of the

potential of plaque as a significant reservoir for fluoride follow. In order to further formulate the studies of this thesis an overview of the approaches used in studying dental caries is included, and focuses primarily on *in vitro* methodology. The strengths and limitations of abiotic and biofilm models is discussed providing rationale for the CDFF model developed in the work of this thesis. A specific review of studies modelling the anti-caries efficacy of fluoride highlights the lack of a suitable *in vitro* biologically relevant biofilm caries model. Subsequently, some chosen techniques for assessing enamel demineralisation and methods for determining biofilm cariogenicity, are presented. No previous studies have tested the correlation between current gold standard, yet destructive, techniques in determining enamel demineralisation with emerging non-invasive ones such as non-contact surface profilometry (NCSP) and quantitative light induced fluorescence (QLF) within a biologically relevant caries model. Finally, the use of QLF in imaging, quantifying and characterising biofilm is discussed; highlighting the extent of its application to date. Relatively little has been known about the significance of biofilm fluorescence but recent studies suggest that it could be used as an indicator of biofilm cariogenicity.

1.2 BIOFILMS

Central to the dental caries disease process is the metabolic activity of biofilm bacteria in breaking down dietary carbohydrates and causing the localised demineralisation of dental enamel. In general terms, a biofilm can be defined as a structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface (Costerton *et al.*, 1999). Beyond this, they are not easy to define summatively since they are extremely complex microbial ecosystems, varying greatly in structure and composition depending mainly on the environment they are situated within. Dental plaque is the diverse microbial

community found on the tooth surface embedded in a matrix of polymers of bacterial and salivary origin (Marsh and Bradshaw, 1995). Developing naturally on teeth, it forms part of the host's defence against colonisation by exogenic, often pathogenic microorganisms though when allowed to accumulate or when exposed to changes in specific environmental conditions, disease can ensue. This literature review, and the overall work of this thesis focuses namely on the highly prevalent plaque related disease; dental caries.

1.2.1 Prevalence and Importance in Humans

An estimated 65% of all hospital infections are of biofilm origin (Donlan, 2001; Donlan and Costerton, 2002; Douglas, 2003; Ramage *et al.*, 2006). They have been found to be associated with medical implants (Donlan and Costerton, 2002) and with diseases such as cystic fibrosis, colitis, urethritis, vaginitis, conjunctivitis and dental plaque (Chernish and Aaron, 2003; Høiby *et al.*, 2005). Dental plaque mediates the development of dental caries. With 60 – 90% of school children suffering dental caries globally, the World Health Organisation reports that oral disease is the fourth most expensive disease to treat in industrialised countries (WHO Factsheet 318, 2012). In 2006, an estimated £1977 million was spent in dentistry for dental treatments and disease prevention, in England alone (NHS Report, 2008). The impact of dental caries and oral health factors is further explored later in this review. In 2007, The Centers for Disease Control and Prevention Report stated "In American hospitals alone, healthcare-associated infections account for an estimated 1.7 million infections and 99,000 associated deaths each year. Of these infections: 32% of all healthcare-associated infections are urinary tract infections; 22% are surgical site infections; 15% are pneumonia infections of the lung; and 14% are bloodstream infections" – all biofilm related (Dye *et al.*, 2007). Evidence of such prevalence further propels continuing research in both general biofilmology and in cariology.

1.2.2 Historical Perspective

The first documented evidence of the existence of microbial biofilms involved observations on what we now term dental plaque. Antonie van Leeuwenhoek, in the seventeenth century, isolated opaque deposits between his teeth "as thick as if it were batter" and coined the term "animalcules" we now know are the bacteria of dental plaque (Dobell, 1932). It was not until the late 1930s – early 1940s that it was shown that organic matter and bacteria could accumulate on solid surfaces. Early pioneers of the concept that microorganisms attach to surfaces investigated the attachment of bacteria in soil to glass slides submerged for specific time intervals (Winogradsky, 1928; Cholodny Kiew, 1930); noting that bacteria able to grow on slides differed from those cultured from those in the water phase. Henrici (1933) found that for the most part freshwater bacteria did not float freely but grew attached to surfaces. All reported that bacterial populations were diverse, attached to surfaces and grew to form complex microbial films. In a review of his key findings regarding bacterial growth on surfaces, ZoBell (1943) observed that when seawater was collected in sterile glass bottles there were more bacteria on the surface than in the free floating planktonic phase with an increase in biofilm microorganisms and a decrease in free-living ones over time (ZoBell and Anderson, 1936; ZoBell *et al.*, 1937). ZoBell concluded that it was likely that the nutrients were concentrated on the solid surfaces and hypothesised that this could mean bacteria were attracted to colonise the surface and their growth and survival was enhanced at these surfaces (ZoBell, 1936). He postulated that by keeping the bacteria, their enzymes and nutrients close to a surface, the microbial film was capable of causing damage to it. His studies also suggested that the adhesion of bacteria consisted of a two-stage process of reversible association followed by irreversible attachment (ZoBell, 1943).

Extensive chemical and physical analysis of bacterial biofilms began in the late 1960s – early 1970s when the prevalence of biofilm was once again noted and due to

advancements in numerous analytical techniques. Applying scanning and transmission electron microscopy, Jones *et al.* (1969) revealed that wastewater biofilms were composed of a variety of different microorganisms situated in a polymer matrix primarily composed of polysaccharides. By 1978, Costerton recognised their ubiquity, coined the term "biofilm" and was able to prove that many bacteria spend the majority of their existence in such communities, so biofilm life was the rule rather than the exception. Using biofilms found in mountain streams and dental plaque, he hypothesised the mechanisms these microorganisms employed to adhere to living and non-living materials and how they derived benefit from these ecological niches (Costerton *et al.*, 1978).

1.2.3 Why Biofilms Form

Compared with existence in a planktonic state, forming biofilm is advantageous to microorganisms; playing a key role in their survival. Microorganisms in a biofilm differ markedly from their planktonic counterparts in terms of physiology, structure and behaviour. Advantages include enhanced access to nutrients; closer proximity between cells facilitates mutualistic or synergistic associations (Marsh *et al.*, 2011), cell-to-cell signalling (Copper *et al.*, 1995), protection, the production of copious amounts of extracellular polymers (Costerton *et al.*, 1987), acquisition of antibiotic resistance genes by plasmid transfer (Waterhouse and Russell, 2006; Roberts and Mullany, 2010); and the increased expression of beneficial genes causing phenotypic changes in colony morphology. Both the pathogenic potential of microorganisms and resistance to antimicrobials has been shown to increase significantly once in biofilm state (Anwar *et al.*, 1989; Gilbert *et al.*, 1997; Lewis, 2001). In fact the idea that biofilm bacteria can possess novel properties in comparison to those in planktonic states was first made when Antonie van Leeuwenhoek reported that dental plaque bacteria were not killed when teeth were rinsed with a strong wine-vinegar yet they were killed when dispersed into solution (Dobell, 1932). The biofilm matrix is

biologically active, preventing desiccation by retaining nutrients, water and key enzymes within the biofilm (Branda *et al.*, 2005). Characklis (1973), studying slimes in industrial waters, was able to demonstrate that biofilms were highly resistant to the antimicrobial effects of chlorine while it was found that biofilm provided a physical/chemical barrier offering some protection against antibacterial agents in pathogenic systems of disease (Costerton *et al.*, 1981). Therefore, the study of microorganisms within biofilm growth states is particularly significant when formulating antimicrobials. They need to account for this increased resistance in an effort to overcome biofilm related diseases such as dental plaque mediated dental caries.

1.3 DENTAL PLAQUE

Dental plaque can be defined as the diverse microbial community found on the tooth surface embedded in a matrix of polymers of bacterial and salivary origin (Marsh, 1992). Though microbial deposits on teeth seem to be the first type of biofilm to be recorded in the literature as early as the eighteenth century, and subsequent descriptions of a "slime layer of denticolae" (Ficinus, 1847 as cited in Küchenmeister (1857), "coatings of leptothrix" (Leber and Rottenstein, 1867) and the observation that a "felt-like mass of microorganisms [was] present where decay had started"(Williams, 1897); it was G. V. Black (1884) who coined the term "gelatinous microbial plaque". Within the human oral cavity most oral microorganisms attach to and grow at surfaces and this ability allows them to persist as resident microflora. The environment of the mouth is highly heterogeneous, providing a variety of unique highly selective microhabitats for microbial colonisation. Despite milieu selectivity, over 700 different types of microorganisms have been identified (Aas *et al.*, 2005) on mucosal surfaces, all parts of the tooth and the gingival crevice. Molecular techniques identified over 630 distinct bacterial species (Kazor *et al.*, 2003) whilst over 3600

unique sequences were found in an individual mouth (Zaura *et al.*, 2009). Supragingival plaque is biofilm developed on tooth surfaces above the gingival margin including biofilm found interdentally. Since this plaque has access to soluble nutrients from the diet and saliva and must withstand abrasive forces associated with chewing and oral hygiene procedures, it is dominated by saccharolytic, facultative and adhesive organisms (Loesche, 1986). The presence in the mouth of a resident microflora makes a significant contribution to host health (Marsh, 1994, 2000) and only when its beneficial properties are disrupted is disease able to ensue.

1.3.1 Stages in the Formation of Dental Plaque: a Model Biofilm

The formation and development of biofilm is a multistage process and dental plaque forms in accordance with sequences found to be the case in most biofilm (Palmer and White, 1997). The development of dental plaque can be sub-divided into a number of phases (Nyvad, 1993) and as plaque formation is a dynamic and continuous process, these phases overlap (Marsh and Bradshaw, 1995).

A. Formation of a complete conditioning film (the acquired pellicle) on the tooth surface (begins immediately and complete by 90 – 120 min).
B. Non-specific reversible phase involving physico-chemical interactions between salivary bacteria and the acquired enamel pellicle (0 – 24 hr).
C. Short-range specific stereo-chemical molecular interactions between primary bacterial colonisers and host receptor molecules in the acquired pellicle.
D. Attachment of secondary colonisers to already attached primary colonisers (co-aggregation). Growth of attached bacteria leads to the formation of distinct micro-colonies (4 – 24 hr).
E. Development of horizontal and vertical stratification within the developing biofilm and increased bacterial succession (1 – 7 days).
F. Growth and the formation of a climax community (1 week and upward).

1.3.1.1 Pellicle formation

Host and bacterial molecules form a conditioning film on the surface of cleaned tooth enamel through a process of selective adsorption. The pellicle contains both saliva derived, and molecules of a bacterial origin. Bacterial components such as glucosyltransferases and glucans have been detected within the acquired pellicle (Scheie *et al.*, 1987; Schilling and Bowen, 1988) while acidic proline-rich proteins; amylase, lysozyme, statherin and immunoglobulins were found to be amongst host molecules using immunological probes (Al-Hashimi and Levine, 1989). In this regard, the pellicle can be viewed as metabolically active. The tooth pellicle is predominately proteinaceous consisting of albumin, glycoproteins, lipids, lysozyme, phosphoproteins and other components of saliva and gingival crevicular fluid (Levine *et al.*, 1985). The components of the salivary fluid environment are a major contributing factor in the composition of the conditioning film. This film forms, and reaches plateau, relatively rapidly (90 – 120 minutes), is usually less than 1 μm thick and allows for the adhesion of oral bacteria. By acting as receptors or adhesins, these molecules therefore possess a selective influence over the pattern of subsequent biofilm development. Key in its role, the complexity of the pellicle leads to modifications of the underlying surface and influences the rate and extent of further microbial adhesion (Mittelman, 1996). It provides a concentrated nutrient source and important trace elements for early colonisers yet it is important to note that conditioning films may inhibit, as well as promote, the adhesion of certain bacteria (Percival *et al.*, 2011). Since the acquired enamel pellicle contains a high level of sulphate and carboxyl groups it can further increase the net negative charge of the tooth surface and since bacteria are also net negatively charged, initial repulsion exists between the two. This forms the basis of an innate defence mechanism which, once broken down, leads to plaque formation.

1.3.1.2 Microbial colonisation & adhesion

Generally, oral microorganisms are transported passively to the tooth surface by the regular flow of saliva, achieved via a number of well-established fluid and thermodynamic processes including mass transport, thermal dynamics such as Brownian motion and molecular diffusion as well as gravitational effects – differential settling and sedimentation, for example (Characklis, 1981). Now that the enamel is coated with pellicle, when cells approach the tooth, the combined effect of long range physico-chemical forces provide a weak, non-specific area of attraction around 10 – 20 nm from the surface leading to weak, reversible bacterial – surface associations (Rutter and Vincent, 1980; Busscher *et al.*, 1992). Specific short-range stereo-chemical interactions can occur increasing the strength of this early reversible attachment if the cell is able to get closer to the enamel surface. This can only happen if water is removed from between the surface of the tooth and the colonising bacteria. It is believed that the dehydrating effects of hydrophobic bacterial cell walls may play a role here, allowing the surfaces closer proximity. Additionally many bacterial adhesins are located on surface structures such as fibrils on streptococci (Handley *et al.*, 1991) and fimbriae on other genera such as *Actinomyces* spp (Rosenberg and Kjelleberg, 1986; Bullitt and Makowski, 1995) that enable bacteria to bind to complimentary molecules in the pellicle in a selective manner (Gibbons, 1989). A number of studies have identified specific molecular interactions to facilitate this phase including the characterisation of interactions with molecules such as statherin alone and in combination (Stromberg and Boren, 1992; Stromberg *et al.*, 1992; Kolenbrander and London, 1993) with fractions of saliva and a high molecular weight acidic proline-rich protein (PRP-1) (Gibbons and Hay, 1988; Gibbons *et al.*, 1988; Gibbons *et al.*, 1991); α-amylase (Murray *et al.*, 1982; Scannapieco *et al.*, 1989; Douglas *et al.*, 1990; Ramasubbu *et al.*, 1996), and lectin-like bacterial proteins associating with pellicle glycoproteins (McBride and Gisslow, 1977; Shibata *et al.*, 1980; Cisar *et al.*, 1981; Murray *et al.*, 1982; Murray *et al.*, 1986).

Initial bacterial colonisers have been found to be mainly *S. sanguinis*, *S. oralis* and *S. mitis* biovar 1 (Nyvad and Kilian, 1990). These account for 56% of the total initial microflora and 95% of the streptococci of oral biofilm, both supra- and subgingivally. *Actinomyces* spp. and Gram negative bacteria such as *Haemophilus* spp. and *Neisseria* spp. are also present within the initial microflora. Li *et al.* (2004) found that in healthy subjects *Actinomyces* spp. were the dominating species after two hours of biofilm formation with *S. oralis* and *S. mitis* prevailing after six hours. Interestingly, where *S. mutans* is often discussed as the main aetiological agent associated with the development of caries (Kreth *et al.*, 2005), *S. salivarius* is often associated with building stability in the biofilm matrix via lactose uptake and urease enzymes (Chen *et al.*, 1996; Sissons and Yakub, 2000) and with this stability prevent colonisation by opportunistic pathogens (Uehara *et al.*, 2001; Tada *et al.*, 2006). Bacteria of the mutans streptococci group were believed to constitute a significant portion of the initial microflora since they could produce elaborate sticky extracellular polysaccharides from sucrose *in vitro* (Marsh and Nyvad, 2008). In fact it was shown that irrespective of an individual's consumption of sucrose, mutans streptococci constituted only 2% or less of the initial streptococcal microflora in a clinical study (Nyvad and Kilian, 1987). This is likely due to the relatively low concentration of these species in saliva and that glucan production from sucrose does not promote the earliest colonisation of tooth surfaces by mutans streptococci. It is now known that *S. mutans* is much less efficient at adhering to enamel surfaces than *S. sanguinis* (van Houte and Green, 1974). After all it was shown that approximately $10^4 – 10^5$ cells of *S. mutans* mL^{-1} of saliva needed to be present before one cell was recovered from a clean enamel surface compared to 10^3 cells mL^{-1} *S. sanguinis* (van Houte and Green, 1974). Co-culturing *Actinomyces* spp. with *Lacobacillus* spp. demonstrated the pivotal role the former plays in biofilm formation (Filoche *et al.*, 2004). Here, *Lactobacillus* spp. species could not form biofilms when inoculated alone but could in the presence of

Actinomyces spp.; further evidence of the microbial complexity of dental plaque even at early stages of biofilm formation.

1.3.1.3 Microbial succession

As time progresses the resident microbial community of dental plaque is gradually replaced by species more suited to the changing physicochemical environment. Pioneer bacteria create environments that are either more favourable to secondary invaders or increasingly less sustaining to themselves due to a lack of nutrients, the accumulation of inhibitory metabolic products, or an increase in anaerobiosis. Adhesin-receptor interactions mean that secondary colonisers can coaggregrate or coadhere to the established pioneer species (Kolenbrander, 2000). It has been documented that *A. naeslundii, S. gordonii*, *S. mitis*, *S. oralis* and *S. sanguinis* coaggregate and that these interactions are highly specific (Kolenbrander and London, 1992) while some are known to act as co-aggregation bridges between non-coaggregating bacterial species (Kolenbrander and London, 1993). It has been suggested that these, specifically *Fusobacteria nucleatum*, act as a bridge between early and late colonisers of the tooth surface. While it is known that fusobacteria do not coaggregate amongst themselves it has been elucidated that they coaggregate with the largest range of bacterial genera (Kolenbrander *et al.*, 1989; George and Falkler, 1992). Coaggregation not only enables the surface attachment of complex bacterial communities but could offer advantages metabolically; for instance, by aiding the breakdown of complex host molecules by allowing cross-feeding amongst the established community (Marsh and Bradshaw, 1995). As dental plaque matures gradients in biologically significant factors occur due to microbial metabolism and these influence the spatial distribution of the bacteria. Gradients in pH, dissolved oxygen and cations develop along with those in essential nutrients and metabolic end-products; leading to a mosaic of micro-environments consisting of vertical and

horizontal stratification within the biofilm (Marsh, 1992). For example, it was shown that after 9 days the community can shift to be predominated by facultative and oligate anaerobes (Ritz, 1967) due to the reduction in the oxygen concentration (i.e. increased anaerobiosis) as the biofilm increased in thickness despite; in essence, existing in an aerobic environment.

1.3.2 Structure and Properties of Dental Plaque: a Model Biofilm

Biofilm structure is highly influenced by the microorganisms present, substratum, available nutrients, pH and temperature. Alterations in any of these aspects affect bacterial growth and the development of the biofilm, in turn affecting biofilm structure. Molecular and imaging techniques have revealed that dental biofilms display properties consistent with those of biofilms elsewhere in nature such as; open architecture, protection from desiccation, enhanced tolerance to antimicrobials, modulated gene expression, cell – cell signalling, spatial and environmental heterogeneity, broad range of habitats, efficient metabolisms and enhanced virulence.

So far we can see that the behaviour of microorganisms on a surface as part of a biofilm can be very different to that observed in the laboratory in planktonic culture. Particularly important for clinical outcomes is the finding that oral bacteria are less sensitive to antimicrobials during growth on a surface and this resistance increases as the biofilm matures (see Section 1.2.3). Both mixed and pure cultures propagate into biofilm forming highly heterogenous structures; often evident as patchy configurations of cell clusters within a matrix of extracellular polymers (EPS). A major constituent of dental plaque is water (approximatately 80 – 90% wet weight) while 70% of the dry weight of plaque is accounted for by bacteria with the remainder comprising a matrix of polysaccharides, salivary proteins and glycoproteins (Marsh and Bradshaw, 1995). Bacteria employ enzymes (glucosyltransferases and fructosyltransferases) to produce extracellular polysaccharides of glucose (glucan with

1, 6-α-linkages; mutan with 1, 3-α-linkages) and fructose (inulin, 2, 1-ß-linkages) from dietary sucrose (Marsh and Bradshaw, 1995). Some glucans can be involved in bacterial adhesion to surfaces and insoluble ones are believed to play a significant role in determining the architecture of the biofilm. Bacteria that are capable of producing the appropriate hydrolases, can breakdown and metabolise some of these polysaccharides; a phenomenon to be discussed more extensively later in this thesis (Section 1.4.3.4).

Once believed to be simple slime layers with little organisation, advances in imaging techniques have revealed that biofilms are highly structured. Fluorescent *in situ* hybridisation determined that biofilm bacteria were located within a matrix of extracellular polymers (Costerton *et al.*, 1995) while the overall structure of biofilm was revealed using confocal scanning laser microscopy techniques (Wood *et al.*, 2000; Auschill *et al.*, 2001). Open channels resolved in biofilm provide further evidence of the spatial and temporal complexities of biofilm and these have been shown to contain few bacterial cells and more permeable matrix material (Lawrence *et al.*, 1991). These channels are often referred to as voids or pores, and facilitate mass transfer, favouring higher nutrient concentrations and allowing cellular metabolites and by-products to be more concentrated under cell clusters (Percival *et al.*, 2011). In this way it is logical that such channels have been likened to a simple open circulatory system in the past (Costerton *et al.*, 1995). Opinions have differed as to whether these channels are fully open or filled with EPS. Calcofluor staining of biofilms of a glucosyltransferase C-deficient knockout mutant of *S. mutans* incapable of forming extracellular matrix, provided evidence that they are indeed filled with EPS (Thurnheer *et al.*, 2006). High molecular-weight dextran passed at a 16-fold increased velocity compared to wild-type strains suggesting the presence of EPS within the channels and that this matrix delays the mass transfer of macromolecules (Thurnheer

et al., 2003). Several studies have observed that the structural organisation in dental plaque is dense and includes microcolonies of similar shaped bacteria, parallel orientated bacteria and coaggregates between bacteria that form "corn cob" structures (Listgarten, 1976; Marsh and Bradshaw, 1995). This last type is evidence of direct interspecies relationships between central filamentous organisms coated with spherical ones mediated by surface fibrils (Nyvad and Fejerskov, 1994). As dental plaque builds in complexity gradients in biologically significant factors are produced and these have a direct influence on the spatial distribution of the bacteria present leading to a variety of microenvironments (Marsh, 1992). Gradients in key factors, such as oxygen, nutrients and pH, can develop over short distances throughout the depth of dental plaque since the free movement of molecules is often reduced by constituents of the biofilm matrix. It has been found that the most viable cells line these voids and channels (Auschill *et al.*, 2001; Hope and Wilson, 2003); and can be attributed to the richer supply of nutrients from the medium flow within these channels (Wimpenny *et al.*, 2000). Furthermore, it was elucidated that a viable outer region exists surrounding a non-viable central portion within bacterial stacks of mature microcosm biofilm using a viability mapping technique (CLSM together with live/dead staining) (Hope *et al.*, 2002).

It has been demonstrated that four major factors influence biofilm structure; surface properties, hydrodynamics, nutrients and biofilm consortia (Stoodley *et al.*, 1997). The automaton model (Wimpenny and Colasanti, 1997) indicates that in the presence of high concentrations of substrates, dense biofilms such as dental plaque form. On the other hand, nutrient limited conditions lead to the formation of a layer of individual cells at a surface from which stacked structures develop. Dense biofilms like dental plaque can be simulated *in vitro* by properly taking into account the role substrate concentration plays. Studies have also shown that DNA from lysed cell

functions form another structural component in biofilms. DNA coated the "corn cob" arrangements of *Pseudomonas aeruginosa* biofilms as a scaffold of the EPS matrix (Allesen-Holm *et al.*, 2006) while experiments with DNAase confirmed the important function of extracellular DNA (Whitchurch *et al.*, 2002) in biofilm structure. This enzyme cleaved DNA and inhibited biofilm formation yet it had no effects on planktonic bacterial growth.

Dental plaque possesses diverse properties that confer bacterial survival at surface interfaces. A vital property of dental plaque is that gene expression is highly modulated during biofilm development and this is achieved via direct and indirect mechanisms. For example, the genes responsible for encoding adhesins can be induced when oral bacteria bind salivary proteins. It was shown that during the first two hours of *S. mutans* biofilm formation 33 proteins were differentially expressed upon attachment. Additionally an increase in the synthesis of enzymes involved in the catabolism of carbohydrates was observed (Welin *et al.*, 2004). By day 3 some glycolytic enzymes were down-regulated and proteins associated with other biochemical functions up-regulated in *S. mutans* biofilms (Svensater *et al.*, 2001).

As biofilm develops there are increasing opportunities for cells to interact with each other via cell signalling systems, and with other species in a range of conventional synergistic and antagonistic biochemical interactions (further discussed in the next Section 1.3.3). Mature biofilm, therefore, demonstrates the complexity of the challenge when attempting to find correlations between the microbial composition of dental plaque and the development of caries; and illustrates how disease may be due to the outcome of many interactions among different types of bacteria.

1.3.3 Microbial Interactions in Dental Plaque

As dental plaque develops and becomes more diverse the biofilm matrix provides a medium for the transfer of genetic material enabling microbial communication and bacterial interaction. A true understanding of cariogenesis will not be achieved without properly considering the rapidly increasing amount of information on the variety of synergistic and antagonistic interactions between species within dental biofilm; especially those dependent on the action of signalling molecules. To ensure their individual survival, bacteria possess programmes of complex gene expression, upregulated when cohabitating in dense populations. In addition to colonisation factors so they can symbiotically interact with the host, biofilm bacteria possess signalling strategies that can coordinate their mutual defense via the production of a number of virulence factors (Spoering and Gilmore, 2006). In health, the stable bacterial climax community prevents colonisation by unwanted, potentially pathogenic, invaders yet since resources within biofilm are limited, there is extensive evidence of high levels of competitive behaviour and cellular interaction within biofilms in natural ecosystems (Connell and Slatyer, 1977; Fredrickson, 1977). Microbial competitive strategies mean that the biofilm is in a constant state of flux (Baier, 1984; Wahl, 1989) as demonstrated by microbial succession (Section 1.3.1.3). Microorganisms invariably interact due to their close proximity in biofilm and interactions may exert beneficial or adverse effects on one another (James *et al.*, 1995; Stewart *et al.*, 1997). Synergistic interactions involve the concerted action of several bacteria, often facilitating the development of food chains (Marsh and Bradshaw, 1995) and the retention of extracellular enzymes within EPS generates a complex external digestive system (Flemming *et al.*, 2000). They can also lead to the co-operative degradation of complex host molecules, such as salivary mucins (Bradshaw *et al.*, 1994). *Veillonella*'s utilisation of lactate produced by the metabolism of sugars by streptococci or actinomyces is perhaps the best-revealed dental plaque food chain. In removing lactate from the environment, this interaction modulates the

demineralisation of enamel by bacterial acids. On the other hand, the production of inhibiting factors such as bacteriocins and hydrogen peroxide by biofilm bacteria bring about antagonistic effects on their neighbours (Marsh, 1992). Without bacterial interactions, the only factor in the survival of species would be their individual growth rates, leading to the survival of limited species in dental plaque; basically only those most metabolically suited. In reality, the complex co-habitation observed in plaque supports that interactions are occurring and are key in achieving such biodiversity.

Quorum-sensing is considered crucial in the regulation of colonisation and virulence factor expression within the biofilm (Yarwood and Schlievert, 2003) and describes microbial cell-to-cell communication using chemical messages (Nealson and Hastings, 1979; Miller and Bassler, 2001). Often referred to as microbial pheromones or autoinducers, the concentrations of the specific chemicals involved often correlates with the density of the microbial population of the biofilm. When large numbers of neighbouring bacteria are sensed in a small environment, bacteria modulate their physiology, for example by slowing down their metabolism. This decrease in metabolic activity is beneficial by preventing the depletion of available nutrients as well as the accumulation of waste products (ten Cate, 2006). Quorum-sensing is governed by different molecular mechanisms in different types of bacterial species. Gram positive bacteria such as streptococci and lactobacilli; major components of dental plaque, have not yet been reported to use autoinducer-based signalling systems; though, in some an oligopeptide pheromone is involved in inter-bacterial signalling (Percival *et al.*, 2011). It has been shown that these bacteria employ signalling through the small peptide, named the competence signalling peptide (CSP) (Havarstein *et al.*, 1996; Cvitkovitch, 2001). When levels of CSP reached a threshold, the lysis of a subpopulation of biofilm bacteria was triggered; in effect alleviating nutrient limitation and providing DNA to the environment which could be taken up

by other competent bacteria (Petersen *et al.*, 2005). Defined as the ability to take up foreign DNA, competence along with the ability to transfer DNA are essential components in exchanging genetic material and may give rise to mutations in the bacterial genome necessary for evolution (Thomas and Nielsen, 2005). CSP has also been shown to control biofilm formation since CSP-deficient mutants possessed less biofilm-formation properties whilst adding synthetic CSP increased the biomass of a variety of streptococcal species (Li *et al.*, 2002). The addition of exogenous CSP induced competence. Quorum sensing in gram negative bacteria such as *Porphyromonas gingivalis* and *Veillonella*, operates via autoinducers, in particular acylated homoserine lactones (AHLs). Much like CSP, when levels of AHLs reach a threshold concentration, changes in gene expression occur (Whiteley *et al.*, 1999). AHL enters bacterial cytoplasm by diffusion, up-regulates the transcription of specific genes to influence the production of vital proteins and eventually influences the structure of the biofilm. Quorum sensing systems have also been shown to be involved in the regulation of virulence in some pathogens. For example, the fimbrial expression of *Porphyromonas gingivalis* was shown to be modulated by *S. cristatus* in dental plaque to prevent its attachment (Xie *et al.*, 2000).

1.4 Dental Caries

Dental caries is the localised chemical dissolution of the tooth surface caused by metabolic events taking place in the biofilm associated with these surfaces as biofilm bacteria metabolise dietary carbohydrates into acids. Developing over time, it is a multifactorial disease involving the interplay between the host teeth and saliva; the biofilm microflora, and the host diet as substrate. Biofilm is a prerequisite for caries lesions to occur but not all biofilm attached to teeth result in clinically visible demineralisation. Minute pH fluctuations occur continuously within the biofilm reflecting metabolic events in response to changing nutritional conditions, such as

exposure to fermentable carbohydrates. Shifts in pH influence the chemical composition of the fluid within the plaque so that the relative degree of saturation of this fluid with respect to minerals important in maintaining the chemical components of the tooth surface is affected. Caries lesions arise when the pH drop results in a net loss of mineral. Thereby, dental caries lesions are a result of an imbalance in the physiological equilibrium between tooth mineral and biofilm fluid (Fejerskov *et al.*, 2008).

1.4.1 Personal, Societal and Economic Burden

The relation of oral health to general health and its impacts on quality of life provide an apt starting point when arguing of the importance that sober investigations of dental caries and other biofilm related diseases should continue. Increasingly, focus has been turning away from the traditional oral disease burden assessment towards assessments of how oral disease and conditions impact on people's lives (Baelum *et al.*, 2008). There is evidence supporting the relationship between oral and general health; severe periodontal disease, for example, is associated with diabetes (Grossi and Genco, 1998). Oral health is integral and essential to general health, it is a determinant factor for quality of life and the psychosocial impact of oral diseases often significantly diminishes quality of life. These diseases restrict activities in school, at work and at home, causing millions of school and work hours to be lost each year around the world (Petersen, 2003). Dental caries is the main cause of oral pain, suffering, disability and tooth loss and remains a major oral disease burden globally. Despite this evidence, its ubiquity and non-life-threatening nature have minimised its significance in overall human health compared to other diseases. Yet the economic burden for the treatment of dental infections can be staggering. Despite great achievements in improving oral health in populations globally, problems still remain in communities around the world, particularly among underprivileged groups in both

developed and developing countries. With 60 – 90% of school children and a vast majority of adults suffering dental caries worldwide, the World Health Organisation reports that oral disease is the fourth most expensive disease to treat in industrialised countries (WHO Factsheet 318, 2012). For example, in 2006, an estimated £1,977 million was spent on dentistry for dental treatments and disease prevention, in England alone [NHS Report (NHS, 2008)]. In the USA, the annual cost of the treatment of caries and periodontal disease rose to approximately $24 billion in 1984 (Gotowka, 1985) from over $11 billion in 1977; the restoration of teeth due to dental decay accounted for 90% of these costs (Douglass and Day, 1979). It was generally appreciated that these figures could be gross underestimates of the economic burden of these diseases since they were based on costs for only the 40 to 50% of the public who regularly visit the dentist and receive treatment (Walden and Wilensky, 1982). In 2004 the expenditure for oral health care in the USA had risen to $81.5 billion (Historical National Health Expenditure Data, 2006). Meanwhile, the European Union had spent a total of €54 billion on oral healthcare in the year 2000 alone (Widstrom and Eaton, 2004). Dental caries is also a most prevalent disease in several Asian and Latin American countries, while it appears less common and less severe in most African countries (Petersen, 2003). However, in light of changing living conditions it is expected that the incidence of dental caries will increase in many developing countries in Africa, particularly as a result of a growing consumption of sugars and inadequate exposure to fluorides. If left to proceed without intervention, cavitated teeth pose further impacts on oral health, such as the development of painful periapical and periodontal abscesses, and endodontic infections. Consequently, if such infections remain untreated, osteomyelitis and cellulitis of the surrounding bone and tissue respectively proceed, and patients are increasingly at risk of septicaemia with direct impacts on general health (Cotti *et al.*, 2011). With current concerns regarding the dependency on antibiotic use for the treatment of endodontic infections and its contribution to antibiotic resistance (Garg *et al.*, 2014),

prevention is key and provides further impetus for research aiming to prevent dental caries and its subsequent diseases.

1.4.2 Early Theories in Caries Aetiology

Evidence of dental caries exists in *Homo sapiens* since the Paleolithic era and a number of accounts have been recorded in the history of ancient civilisations. Dating to approximately 5000 BC, a clay tablet found in Ninevah, Lower Mesopotamia, now housed in the British Museum could be the earliest explanation and description of therapy for toothache. This Sumerian text speaks of tooth decay and aches as caused by worms satiating themselves by "suck[ing] the blood of the tooth…and gnaw[ing] its roots at the gum"; treatment was via a "magic ritual: Mix together beer, the sa-kil-bir plant and oil. Then repeat the magic formula thrice and place the mixture on the tooth" (Prinz, 1909; Ehrlich *et al.*, 2008). Many imagined that the tooth worm bore holes into the tooth and hid beneath its surface surreptitiously, wriggling to cause pain thus a legend was born (Figure 1.1). While there is evidence that around 2700 BC the Chinese used acupuncture to treat pain associated with dental caries, a great surgeon of the Middle Ages, Guy de Cahuliac (1300 – 1368) advocated a cure by fumigation with seeds of leek, onion and hyoscyanus (Newbrun, 1983). Fumigation devices remained in use late into the nineteenth century. The role of worms as a causative of dental decay became accepted in Western Civilisation until 1728 when the father of modern medicine, Pierre Fauchard, successfully challenged this hypothesis, noting the detrimental role of sugar (Ring, 1971). In the meantime, ancient Greeks believed that an imbalance of the four elemental fluids of the body; the "humors", caused all disease including caries (Newbrun, 1983). Additionally, Hippocrates and Aristotle (500 – 300 BC) both observed that the stagnation of food, became putrefied and caused damage; and theorised that both local and systemic factors were related to the cause of caries. Towards the end of the eighteenth century

and remaining dominant until the middle of the nineteenth century, the "vital theory" proposed that caries, like bone gangrene, originated within the tooth itself (Newbrun, 1983). This theory gained favour since it became common that some caries was characterised by extensive penetration into the dentine and the pulp without detectable demineralisation at the tooth surface. This was later opposed on the basis that caries occurred in natural teeth when used in artificial dentures (Magitot, 1867). Soon the role of acid was elucidated when dilutions of nitric and sulphuric acid were shown to dissolve enamel and dentine by Robertson (1835) and by Regnart, (1938) following the theory proposed by Parmly (1819) wherein an unidentified "chymical agent" was responsible for caries. He believed that caries could occur on enamel in locations where food putrified and acquired sufficient dissolving power to produce the disease chemically (Newbrun, 1983).

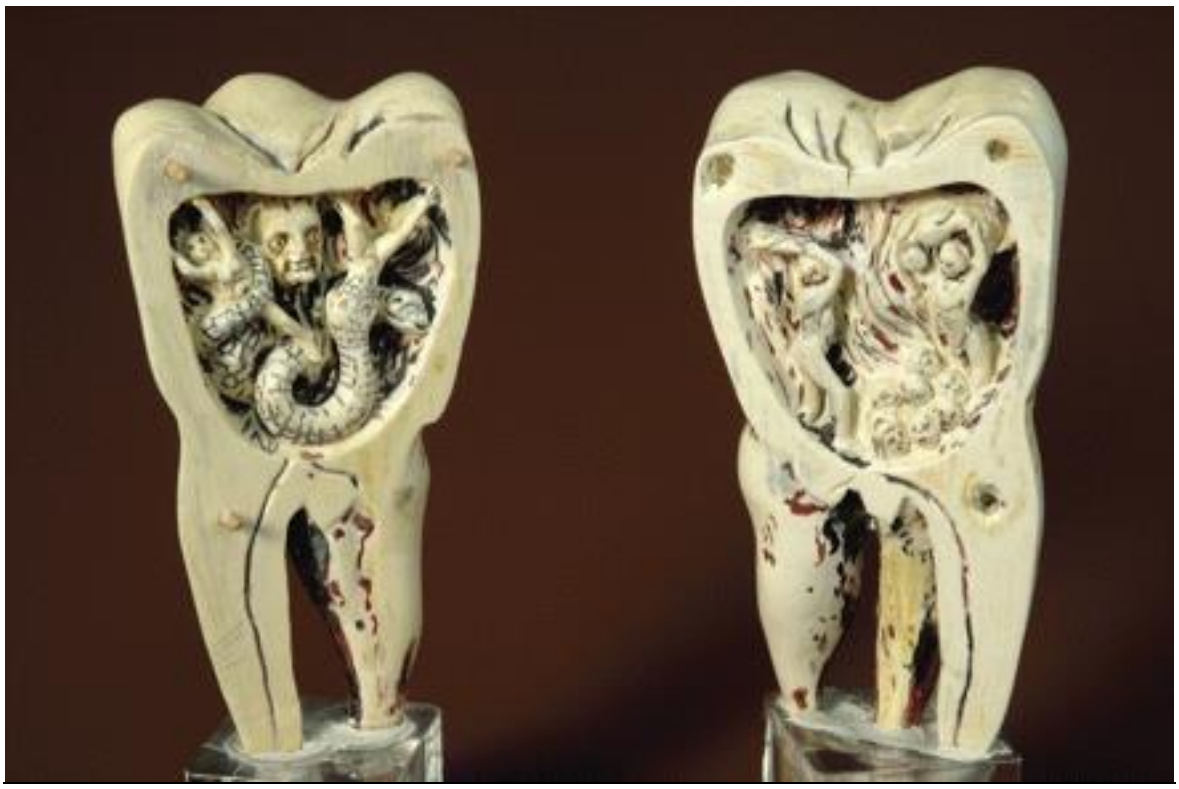


Figure 1.1. "Zahnwurm," (tooth-worm) by anonymous French artist, 18th century, carved ivory image of a human tooth. Collection of the Deutsches Medizinhistorisches Museum, Ingolstadt, Germany (Grizzard & Grizzard, 2011).

Historically, the most significant increases in the prevalence of caries have been associated with dietary changes (Suddick and Harris, 1990), firstly by the increased consumption of plants containing carbohydrates during the Neolithic era (Richards, 2002); as well as the cultivation of rice in South Asia. Caries rates rose sharply in the Middle Ages (Keene, 1980) when the accessibility of sugar cane in the western world improved. Evidence exists of a rise in caries when contact between native North Americans and colonising Europeans was established; believed to be due to an increased reliance on the agriculture of maize (Keene, 1980). Another sharp increase in prevalence was observed around 1850 when widespread increases in the availability of sugar cane, refined flour, bread, and sweetened tea correlated with a greater number of pit and fissure caries.

As mentioned in an earlier section (Section 1.2.2.), what we now term bacteria have been observed in dental plaque as early as the seventeenth century and in 1843, Erdl described filamentous parasites in the "surface membrane" of teeth followed by Ficinus' observations of "denticolae" in material taken from carious cavities. He implied that bacteria cause the decomposition of the enamel, then dentine but neither suggested an explanation of how; therefore this theory has come to be referred as the "parasitic or septic theory". The theories that underpin and acknowledge the role of microbiology in caries and the studies that provide evidence and understanding of plaque biofilm bacteria will be explored in the next section.

1.4.3 The Role of Microbiology in Caries

Though there has been considerable debate about which microorganisms produce caries lesions and how they do it, there is consensus that it cannot occur without the presence of microorganisms (Newbrun, 1983; Tanzer *et al.*, 2001). The "chemo-parasitic" theory of caries credited to Miller (1890) married the concepts of the

chemical and parasitic theories to postulate that "two factors always have to be in operation; the action of acids and the action of germs" for caries to develop. Building on Pasteur's early observation that microorganisms transform sugars into lactic acid by fermentation; and that fermentation of sugars caused the dissolution of tooth mineral *in vitro* (Magitot, 1867); Miller's experiments and writings helped establish this concept firmly. Artificial lesions similar to caries were produced when sound adult teeth covered by wax, except for a small opening, were exposed to dilute acids or fermenting mixtures for an extended period of time (Magitot, 1867; Newbrun, 1983). Leber and Rottenstein (1867) also implicated acids as making enamel porous and identified a specific microorganism, *Leptothrix buccalis*, as the causative agents of caries. They cultured this microorganism from the tubules of carious dentine and believed that it may be responsible for enlarging the tubules and facilitating the rapid penetration of acids. Underwood and Miles (1881) considered caries to be absolutely dependent on the presence of microorganisms when they recovered micrococci, oval and round bacteria in histological sections of carious dentine (Newbrun, 1983). Interestingly, Miller came to the conclusion that no single species of microorganism caused caries and that the process was mediated by any oral microorganism that could produce acids and digest protein. He identified that dental decay is a two stage process of decalcification or softening of the tissues followed by dissolution of the softened residue (Miller, 1890). The chemo-parasitic theory gained further weight when Williams (1897) observed dental plaque on the surface of enamel (Williams, 1897c, b, a). Here plaque was found to partially prevent the dilution and neutralisation of bacteria derived acids by the action of saliva, and was considered to be a means of localising these organic acids formed by bacteria in contact with the tooth surface.

In conflict with the classical chemo-parasitic theory, the "proteolytic" theory was proposed believing that the initial pathway of invasion by microorganisms was via the organic or protein elements of enamel. This theory assumed that because the organic constituent is most vulnerable to the hydrolytic enzymes of plaque bacteria and is degraded before the loss of the inorganic, mineral, phase; caries is initiated in this way. It has been suggested that caries initiation could be attributed to the action of proteolytic enzymes in breaking down lamellae, rod sheaths, tufts and the walls of dentinal tubules (Gottlieb, 1944) and that the caries process involves a proteolytic process of depolymerisation and liquefaction of the organic matrix of enamel (Newbrun, 1983). Frisbie (1944) hypothesised that the less soluble inorganic salts favour solution by acidogenic bacteria once they were dissociated from their "organic bond" and that protein loss allowed widening paths of penetration. Additionally, it was suggested that loosened prisms would fall out mechanically once protein elements, such as the dental cuticle, were broken down by proteolytic organisms (Pincus, 1949). An extension of the basis of this theory came when it was suggested that the breakdown products of organic matter have chelating properties and are capable of dissolving mineral in enamel leading to the simultaneous destruction of both components. Termed the "proteolysis-chelation" theory, decalcification was thought to be mediated by complexing agents such as acid anions, amines, amino acids, peptides, polyphosphates and carbohydrate derivatives (Newbrun, 1983) and happened at neutral pH. Supporters of this theory have also suggested that by interfering with the activity of proteolytic bacteria, acids may prevent tooth decay; speculating that acids may protect organic matter (Schatz and Martin, 1955). Since the proteolysis-chelation theory has a lack of supporting data, it has been seriously questioned in the literature (Bibby *et al.*, 1958; Jenkins, 1961). However, it has subsequently been shown that solutions of amino acids and lactate at approximately neutral pH caused the depletion of calcium in enamel (Onose and Sandham, 1976) and the uptake of phosphorous (Morch *et al.*, 1971). Morch *et al* (1971) went on to

devise a relatively balanced hypothesis that when the pH of plaque is low demineralisation is initiated according to acid dissolution and the caries process is continued by chelated complexes once plaque pH returns to neutral.

Unlike theories related to proteolysis, the chemo-parasitic theory has relatively strong evidence supporting the involvement of acids in the caries process. It has been shown that the pH of smooth surface dental plaque dropped from approximately 6.5 to 5 within two to four minutes of rinsing with glucose or sucrose solutions but that the original pH was gradually restored within 40 minutes (Stephan, 1940). Further work involving continuous and direct measurements of interproximal plaque pH *in situ* at the interface between the tooth and plaque showed that the persistence of acidity may last up to two hours and that the pH drops lower than first anticipated (Graf and Muhlemann, 1966; De Boever *et al.*, 1969; de Boever and Muhlemann, 1969). Specific chromatographic techniques have identified a number of organic acids in dental plaque, bacterial cultures isolated from dental plaque and in caries lesions including; lactic, acetic, propionic, formic and butyric acids (Newbrun, 1983). Quite expectedly, the relative concentrations of these acids vary since dental biofilm comprise a highly mixed bacterial population of homo- and heterolactic fermentative microorganisms, as well as mixed acid fermentative microorganisms that differ in their fermentative capabilities (Newbrun, 1983). Since the proportions of bacteria within dental biofilm are in a state of dynamic flux the relative proportions of the end products they yield are not constant. Variables such as substrate type and concentration, pH and oxygen tension influence the rate of end product formation.

1.4.3.1 Cariogenic features of dental plaque bacteria

The bacteria of dental biofilm can possess a number of cariogenic features including, the ability to rapidly transport fermentable sugars in competition with other plaque

bacteria and ferment them into acids; the ability to maintain their metabolism of sugar under extreme environmental conditions such as when exposed to low pH; and the ability to produce intracellular (IPS) and extracellular polysaccharides (EPS) (Loesche, 1986). It has been found that plaque organisms form a hierarchy with respect to a variety of putative cariogenic traits including acidogenesis and acid tolerance (van Houte, 1980).

1.4.3.2 Dental plaque acids and sugar metabolism

Prior to the 1980's little was known about the biochemical capabilities of dental plaque on a whole, since most studies investigated the metabolic potential of pure strains of microorganisms. Stephan's observation (1944) of the "Stephan curve" heralded new understanding of the pathogenesis of dental caries and sits at the centre of a considerable amount of caries-related research focusing mainly on the acid production phase it elucidated. Findings of this research avenue will be reviewed here. A discussion of the role of alkali generation in plaque physiology follows in Section 1.4.3.5.

With a dissociation constant of 74.1×10^{-5}, lactic acid is a much stronger acid than acetic (1.8×10^{-5}) or propionic acid (1.3×10^{-5}) at the same concentration, temperature and pH. Therefore, it is clinically most effective in demineralising enamel. It has been reported that the concentration of lactic acid was similar to or less than that of either acetic or propionic acids within plaque that had been incubated *in vitro* for five hours (Gilmour and Poole, 1967). The total lactic acid production in plaque exceeded total volatile acids with acetic acid levels shown to be greater than propionic acid and only trace n-butyric acid detected after sucrose consumption (Geddes, 1972). Volatile acids, particularly acetic, then account for most of the total acid in plaque several hours after sucrose was consumed (Geddes, 1973).

Biofilm bacteria employ a number of metabolic pathways to form acid end products from hexose sugars (Figure 1.2); most commonly employing glycolysis primarily. Each six-carbon sugar (C_6) is converted into two molecules of pyruvate (C_3) and a host of end products are derived from this acid depending on the type of fermentation pathway employed by each bacterial species and the conditions of its environment. Homolactic fermenters convert one molecule of glucose into two molecules of lactic acid whilst heterolactic fermentation occurs via the phosphoketolase pathway to produce carbon dioxide and ethanol as well as lactic acid. Homo-fermentative lactic

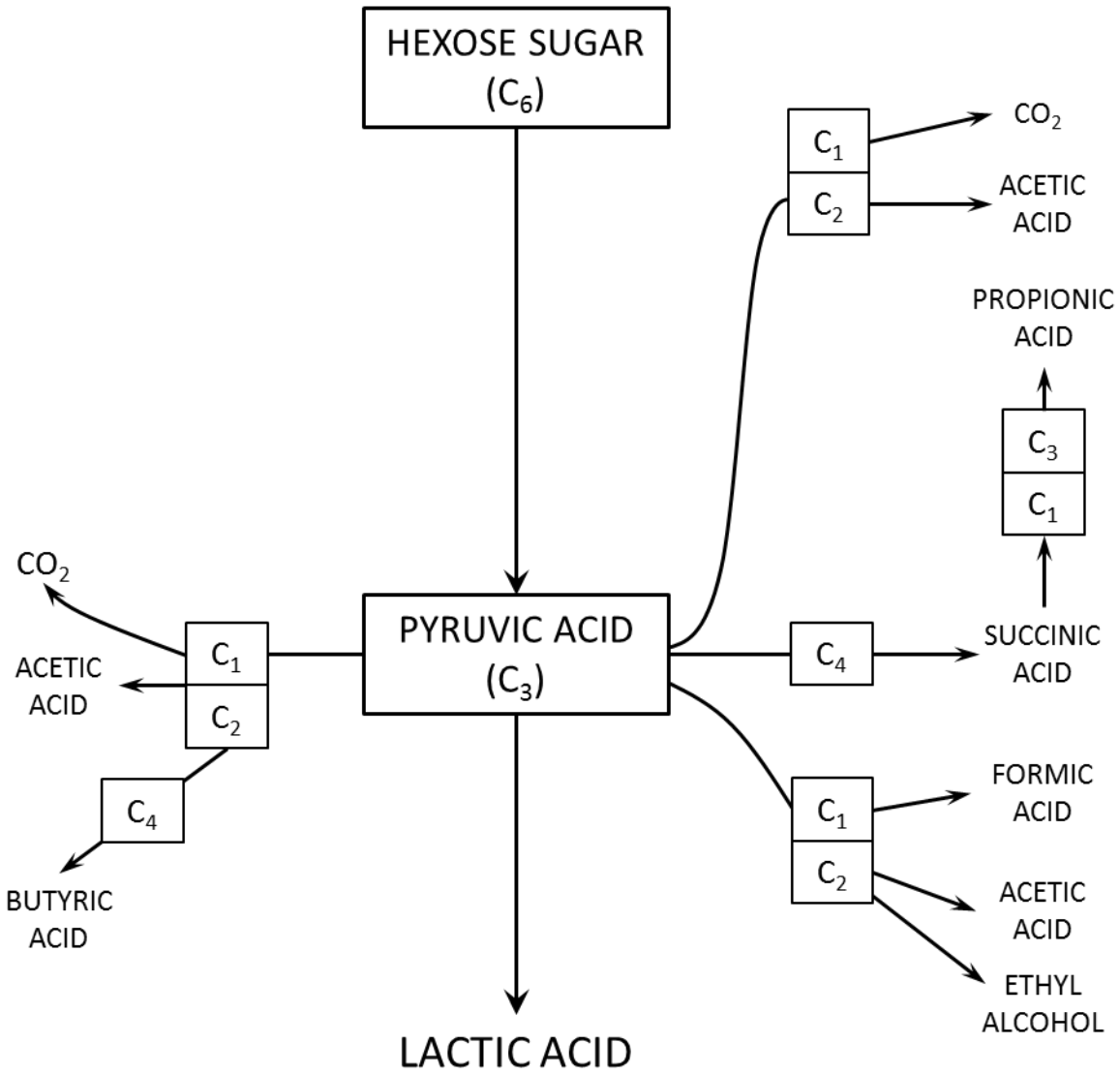


Figure 1.2. Simplified pathway of acidic end products derived from hexose sugars in different glycolytic bacterial fermentations. Intermediate steps and other end products omitted. Adapted from Newbrun, 1983.

acid formers, such as *S. mutans* (Tanzer *et al.*, 1969), convert about 90% of the pyruvic acid into lactic acid mainly via the Embden-Meyerhof glycolytic pathway while converting the remainder into other acids, carbon dioxide and ethyl alcohol (Yamada *et al.*, 1970). When grown in liquid culture, some non-cariogenic oral streptococci produce the same acidic fermentation end products at the same amounts as those shown to be cariogenic (Jordan, 1965; Drucker and Melville, 1968) but on solid media acid accumulation by *S. mutans* is substantially greater than by *S. sanguinis* or *S. mitis* (Plasschaert *et al.*, 1972). These studies suggest that its ability to accumulate high levels of acid may be an important factor in the cariogenicity observed by *S. mutans*.

Lactobacillus spp. are proportionately minor components of healthy dental plaque and capable of either homofermentative or heterofermentative sugar metabolism. The latter type forms less than 90% lactic acid and more acetic and other acids. Propionic acid is formed by a diverse group of plaque bacteria such as, propionibacteria (gram-positive anaerobic rods), peptostreptococci (gram-positive anaerobic cocci) and *Veillonellae* (gram-negative anaerobic cocci), via fermentation. *Neisseriae*, gram-negative cocci, are capable of degrading glucose, pyruvate and lactate into acetate. In air, *Actinomyces* mainly form acetate and carbon dioxide when metabolising glucose. They employ a homolactic fermentation pathway under anaerobic conditions without carbon dioxide, yet in its presence they are able to form formate, acetate, lactate and succinate via a heterolactic pathway. Both *S. mutans* and *S. sanguinis* grown in pure culture produce volatile end products (Carlsson and Griffith, 1974) when under nitrogen or glucose limitation. In continuous culture, *S. mutans* produces various fermentation products in addition to lactate. In starved plaque, when glucose was limited, the main products of the fermentative process were ethanol, acetate and formate (Carlsson and Griffith, 1974). When only nitrogen was limited and glucose

was plentiful; or when the growth of *S. mutans* was rapid; lactate became the foremost fermentation product whereas volatile fatty acids predominated when growth rates were slow (Mikx and van der Hoeven, 1975). A proposed scheme for the regulation of glucose metabolism by *S. mutans* during continuous growth under glucose excess and limitation is shown in Figure 1.3 (Newbrun, 1983).

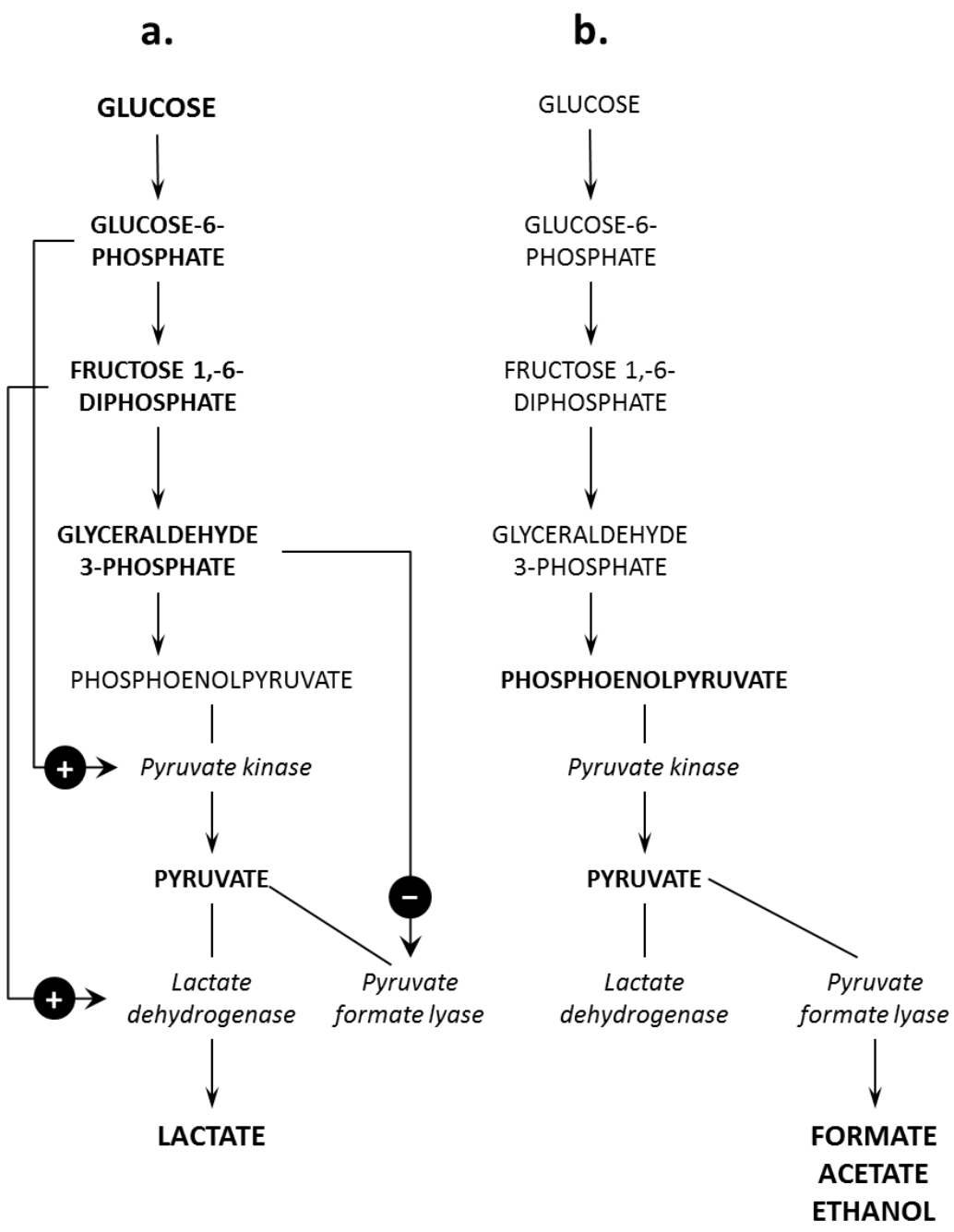


Figure 1.3. Schematic for the regulation of glucose metabolism by *S. mutans* during continuous growth under glucose (a) excess and (b) limitation. +, activation; –, inhibition. Bold font denotes intracellular intermediates at high relative concentrations; and italics denote enzymes. Adapted from Newbrun, 1983.

When *S. mutans* is growing in the presence of excess glucose, pyruvate formate-lyase, responsible for the formation of formate, acetate and ethanol is inhibited by d-glyceraldehyde 3-phosphate and fructose-1,6-disphosphate levels increase, favouring lactate dehydrogenase activity and in turn, lactate formation (Yamada and Carlsson, 1975). In cases where glucose is limited, the intracellular concentration of glucose 6-phosphate is low and this inhibits the activity of pyruvate kinase. The subsequent increased level of phosphoenolpyruvate facilitates the effective transport of glucose into the cell. Here pyruvate formate-lyase activity is raised (Yamada *et al.*, 1976) leading to the predominant production of formate, acetate and ethanol. The presence of such an array of volatile end products along with lactate in dental plaque is an understandable observation. It has been demonstrated that *Veillonella* species are able to form propionate, acetate and carbon dioxide from non-volatile lactate (Mikx and van der Hoeven, 1975; Distler and Kröncke, 1980) and *Neisseria* isolates can degrade lactate to acetate and carbon dioxide via pyruvate and lactate oxidase under aerobic conditions (Hoshino *et al.*, 1976), inherently modulating overall plaque cariogenicity. Furthermore, the pH of dental plaque may be restored to neutrality through the formation of amines or ammonia. The mixed flora can decarboxylate amino acids such as arginine, aspartic, and glutamic acids, histidine, lysine and ornithine, to form carbon dioxide and the respective amine (Hayes and Hyatt, 1974). *Fusobacterium nucleatum*, *Prevotella melaninogenica*, *Treponema* species, peptostreptococci among others can ferment amino acids and release ammonia along with volatile end products such as butyrate and acetate (Newbrun, 1983). The formation of propionic acid involves a more complex pathway wherein oxaloacetate (C_4) and succinate intermediates are formed via carbon dioxide fixation (Figure 1.2). Lactic acid formed by homofermenters can also be degraded forming acetic and propionic acids which are comparatively weaker.

1.4.3.3 Carbohydrate transport systems

Microorganisms are capable of a staggering level of metabolic versatility; most can use a range of carbon sources for energy in response to continuously changing environments and nutrient limitations. *S. mutans* devotes almost 15% of its genome to genes associated with various transport systems (Ajdic *et al.*, 2002), reflecting its evolution as an obligate parasite that is dependent on its host for the provision of many molecules (Russell, 2008). At the time of its publication the *S. mutans* genome had more predicted sugar transport systems than any other sequenced bacterium (BLAST, assession no. AEO14133). Since then, gut-living species of *Lactobacillus* and *Bifidobacterium* have been found to have even more. Sugar substrates are translocated into bacterial cells most commonly by phosphoenolpyruvate (PEP)-sugar phosphotransferase systems (PTSs) but also by ATP-binding cassette (ABC) transporters and galactose-pentose hexuronide (GPH) translocators. PTS systems have been studied extensively, with over 400 papers published on the topic since 1985, and many more before then (Postma *et al.*, 1993). Numerous sugar substrates are bound, translocated across the bacterial membrane and phosphorylated by PTSs; yet these systems also play key roles in the regulation of various metabolic and transcriptional processes within the cell (Dills *et al.*, 1980; Vadeboncoeur and Pelletier, 1997). The translocation of a carbohydrate via these systems across the membrane is coupled to its phosphorylation; with energy for the reaction provided by the glycolytic intermediate PEP.

The proteins that make up PTSs are either general translocators, meaning they are carbohydrate non-specific (enzyme I; EI and histidine protein; HPr), or more specialised to translocate specific substrates (enzyme II; EII). EI and HPr are soluble, cytoplasmic proteins involved in all PTS processes whilst EII are multiprotein permeases which may consist of a single membrane-bound protein with three domains

(A, B, and when present, C and D). In most cases it has been found that EIIC and EIID act as a membrane channel whereas EIIA and EIIB are located in the cytoplasm (Vadeboncoeur and Pelletier, 1997). ABC transporters have common global characteristics and are widespread in bacteria. In gram-positive bacteria, they are typically composed of two permease membrane proteins, two peripheral proteins responsible for binding and hydrolysing ATP; and an extracytoplasmic solute-binding receptor. The receptor domain can be a protein associated to the cell surface bound to the external membrane by electrostatic interactions or a lipoprotein anchored to the cytoplasmic membrane. GPH translocators catalyse the intake of sugars in symport with monovalent cations such as H^+, Na^+ and Li^+, in this way are driven by electrochemical potential and it has been shown that the PTS regulates most GPHs (Poolman *et al.*, 1996). This type of transporter exhibits specificity for ß-galactosides, lactose and both anomers of galactose in a number of bacteria (Reizer *et al.*, 1994).

The capacity for *S. mutans* to rapidly transport and metabolise a wide range of common dietary sugars as they become available has been extensively demonstrated (Ajdic *et al.*, 2002; Ajdic and Pham, 2007; Ajdic and Chen, 2013), could ensure its survival in dental plaque and is therefore a key cariogenic feature of this species. Employing genome sequence analysis, fourteen PTS systems and four ABC sugar transporters have been found in *S. mutans* UA159 (Ajdic *et al.*, 2002) along with the well-described multiple-sugar metabolism (MSM) system (Russell *et al.*, 1992; Tao *et al.*, 1993a; Tao *et al.*, 1993b). A bulk of the sugars tested, but specifically monosaccharides, disaccharides, ß-glucosides and sugar alcohols, were transported by PTS transporters. Five of these transporters were highly expressed regardless of sugar source, presumably so they are available for immediate uptake of sugars consumed; while these studies also provided evidence that glucose, fructose, maltose and sucrose may be preferred sugars for *S. mutans* (Ajdic and Pham, 2007). Inducible

PTSs were also demonstrated with high specificity for particular sugars due to low transcription found in the absence of these sugars, and high transcription in their presence; another indication of the complex adaptation for survival in this species. PTS transport of galactose has not been previously reported to be present in *S. mutans*, it is possible that this sugar is transported by an ABC or a GPH transporter (Ajdic and Pham, 2007). In the presence of sucrose the transcription of ABC transporters was particularly low indicating that they play a minor role in the translocation of this important carbohydrate (Ajdic and Pham, 2007). ABC transporters seem to specialise in the transport of oligosaccharides in this species.

1.4.3.4 Intra- and extracellular polymeric substances

Both intracellular polysaccharides and extracellular polymers (IPS and EPS respectively) play an important role in the cariogenicity of dental plaque biofilm. Their importance in *S. mutans* cariogenicity was confirmed when therapeutic agents that reduce their concentrations in biofilms significantly lessened the incidence of dental caries in rats (Koo *et al.*, 2003; Koo *et al.*, 2005). Originally simply termed "extracellular polysaccharides", EPS was quickly renamed when it became clear that it contained a plethora of biological molecules including, proteins, nucleic acids, lipids and DNA.

A number of studies demonstrate that the metabolism of intracellular storage polysaccharides is a significant trait in cariogenic bacteria primarily by influencing the acidogenicity and resting pH of dental plaque. This consequently probably selects for cariogenic microorganisms to preferentially proliferate within the biofilm microcosm. Early literature showed clear associations between dental caries in animals and humans with the presence of these storage polysaccharides (Gibbons and Socransky, 1962; Loesche and Henry, 1967; van Houte *et al.*, 1969; Tanzer *et al.*, 1976;

Ashley and Wilson, 1977b; Zero *et al.*, 1986b; Spatafora *et al.*, 1995). An *S. mutans* mutant with the ability to produce elevated amounts of IPS was significantly more cariogenic than its wild type counterpart in animal models (Harris *et al.*, 1992; Spatafora *et al.*, 1995). IPS are glycogen-like storage polymers with α-1, 4 and α-1, 6 linkages forming high molecular weight molecules. As an intracellular source of carbohydrate, IPS can be metabolised during periods of nutrient deprivation producing organic acidic by-products in the process (Hamilton, 1976; Tanzer *et al.*, 1976; Zero *et al.*, 1986b). The formation of dental caries can therefore be promoted by the metabolism of IPS since they maintain a lower fasting pH in the plaque matrix, prolonging the period of acid production and the dissolution of enamel by these acids (Tanzer *et al.*, 1976). *In situ* studies have shown pronounced and prolonged decreases in pH and enhanced enamel demineralisation when *S. mutans* produced acid from these intracellular stores (Zero *et al.*, 1986b; van Houte *et al.*, 1989). In addition, *in situ* biofilm exposed to at least 20% sucrose significantly lowered biofilm pH in fasting periods when compared to biofilm with no such exposure (Pecharki *et al.*, 2005; Ribeiro *et al.*, 2005; Aires *et al.*, 2006) and those in the presence of combinations of glucose and fructose along with sucrose elicited higher concentrations of IPS than those without (Tenuta *et al.*, 2006). Concentrations of IPS were at least maintained and even increased over time when studied over a 14 day period of biofilm formation (Paes Leme *et al.*, 2006).

The ability to produce EPS from sucrose is a key cariogenic feature in the survival of some dental plaque bacteria and therein contributes to their pathogenicity and the progression of dental caries. The advantages it bestows are multifaceted including; bacterial adherence (Hamada and Slade, 1980; Rolla, 1989; Schilling and Bowen, 1992), accumulation on tooth surfaces (Freedman and Tanzer, 1982; Larrimore *et al.*, 1983) and the storage of extracellular carbohydrates used as a nutrient source during

periods of exogenous nutrient limitation (Wood, 1967; Gibbons, 1968; Manly and Richardson, 1968; Bowen and Koo, 2011). By promoting cohesive forces, increasing absorption of nutrients and heavy metals (Bryers, 1984), sequestering microbial products, protecting immobilised cells from environmental changes and providing a platform for intercellular communication and gene transfer, EPS exerts many beneficial effects on biofilm (Characklis and Cooksey, 1983). Upon adhesion to a surface, biofilm bacterial cells undergo phenotypic changes, altering many of their structural molecules while derepressing EPS synthesis (Davies *et al.*, 1993). As an organic matrix rich in glycoproteins, proteins and nucleic acids (Humphrey *et al.*, 1979), the EPS of biofilm is thought to account for anywhere between 50 and 90% of its total organic carbon (Flemming *et al.*, 2000) or 10 – 20% of its dry weight (Marsh and Bradshaw, 1995). More recently it has been reported that EPS matrix forms 75 – 80% volume of the biofilm (Socransky and Haffajee, 2002) and that 40% of the dry weight of oral biofilm is polysaccharide in nature (Paes Leme *et al.*, 2006). Sugars such as glucose, fructose, galactose, rhamnose, mannose, N-acetylglucosamine, galacturonic acid, glucuronic acid, guluronic acid and mannuronic acid are all classic constituents of bacterial polysaccharides (Christensen, 1989). The matrix may also contain organic debris, phages, lysed cells, precipitated minerals and other particulate materials. The ability of *S. mutans* to produce large amounts of extracellular, sticky glucans from sucrose was originally considered an important part of plaque formation (Gibbons and Nygaard, 1968; Gibbons, 1989). It is now considered that EPS provides a more important contribution to the pathogenic properties and structural integrity of oral biofilm and that glucan production is an essential expression of virulence by mutans streptococci. One study observed that the demineralisation of enamel was greatest when an artificial biofilm analogous to dental plaque consisted of 95% EPS and only 5% bacteria when comparing the effects of lower ratios of EPS to *S. mutans* quantities (Zero *et al.*, 1986a). Additionally, a dramatic reduction in the ability of mutans streptococci to initiate caries in an experimental animal model was observed

when the genes expressing enzymes responsible for glucan production were deleted (Yamashita *et al.*, 1993). Importantly, differential expression of the polysaccharide synthesis pathway of *S. mutans* using gene fusions to the gene promoter region of the glucosyltransferase B, C genes was demonstrated when bacteria were in biofilm (Burne *et al.*, 1997), compared to planktonic growth phases. Since it has been shown that it does not affect sugar or acid diffusion within the biofilm (McNee *et al.*, 1982; Dibdin *et al.*, 1983) EPS enables sugars to penetrate deeper into the biofilm, increasing the cariogenic challenge at the enamel surface. In thick plaque this penetration and the significant reduction of bacterial buffering elicits a more severe pH fall at deeper portions of the biofilm and acidogenic bacteria produce more acids at the biofilm interface with the enamel substrata (Dibdin and Shellis, 1988; Shellis and Dibdin, 1988). There is still more evidence that EPS plays a role in bacterial adhesion. It has been shown that pre-formed polymers interact with receptors in the acquired enamel pellicle. While ZoBell (1943) suggested the involvement of extracellular 'cementing' substances in the adhesion of cells to the substratum, Corpe (1970) demonstrated the involvement of acidic polysaccharides in bacterial adhesion, and this was confirmed using electron microscopy (Fletcher and Floodgate, 1973). Of 27 bacterial strains tested, those expressing high EPS production showed greater adhesion onto glass beads (Tsuneda *et al.*, 2003). It was suggested that this was due to the polymer interacting between the bacteria and the surface like a tether. When the polymer has high affinity to the surface in question, such steric interactions can cause strong attractive bridging at this interface.

The action of the glucansucrases, glucosyltransferases (GTFs) and fructosyltransferase (Ftf) is responsible for the production of EPS by biofilm bacteria that can metabolise sucrose in this way. GTFs enzymatically catalyse the formation of a growing α-glucose chain by transferring glucosyl units formed from cleaving

sucrose (Henrissat, 1998). The fructose moiety of sucrose is incidentally freed during this cleavage step and Ftf uses it in the synthesis of the EPS fructan (Gibbons and Houte, 1975; Hamada and Slade, 1980). Different sizes and structures of the EPS glucan are produced by three different GTFs expressed in *S. mutans*; GtfB, GtfC and GtfD. The water solubility of EPS is determined by the nature of the linkages between glucosyl units within the chain (Aoki *et al.*, 1986; Hanada and Kuramitsu, 1988, 1989). When the content of α-1, 3 linkages is low and the level of α-1, 6 is high EPS is more soluble and termed dextran (Kuramitsu, 1975; Monchois *et al.*, 1999), whereas if these proportions are inversed mutan is formed; a water-insoluble glucose polymer. GTFs are also able to transfer glucose to fructose and enable the formation of sucrose isomers (Monchois *et al.*, 2000). *S. mutans* biofilms were less cariogenic in animals when mutants in the *gtf* genes were induced than in the parent strains (Johnson *et al.*, 1977; Yamashita *et al.*, 1993) while a study wherein mutants defective in the regions encoding GTFs and FTFs were compared to wild-type, the latter produced more water insoluble glucan while allowing faster diffusion of hydrogen ions (Hata and Mayanagi, 2003). Moreover, the activity of caries in children and EPS synthesis was proposed to be related in clinical studies (Mattos-Graner *et al.*, 2000; Nobre dos Santos *et al.*, 2002). The frequency of sucrose exposure and its concentration also appear to increase EPS concentration in biofilm matrices, lowering pH during fasting periods and enhancing the demineralisation of enamel (Cury *et al.*, 1997; Ribeiro *et al.*, 2005; Aires *et al.*, 2006). Furthermore, the expression of glucosyltransferases by *S. mutans* was significantly up-regulated in older biofilms, yet this was assumed to be caused by the indirect effects of biofilm formation such as nutrient limitation and reduced pH (Li and Burne, 2001).

Finally, it has long been posited that EPS can serve as an exogenous nutrient source. Some components of EPS are only slowly biodegradable and, due to their complexity,

a wide range of enzymes is required for its complete degradation. There is no single enzyme or combination of enzymes that is capable of degrading all polysaccharides within the biofilm matrix (Flemming *et al.*, 2000). Many extracellular enzymes detected in biofilm are involved in the breakdown of extracellular polymers with specific substrates found to include; water-soluble polymers such as polysaccharides, proteins and nucleic acids; as well as water-insoluble compounds such as low-molecular-weight products taken up by bacterial cells and used as sources of energy and carbon. Dextran, inulin and levan formed by oral streptococci are degraded by various enzymes that may be from the bacteria that produced the polymer or from other species within the microbial consortia (Russell, 2009). Though their action is generally slow, hydrolases and lyases are the most significant enzymes to degrade exopolysaccharides (Laue *et al.*, 2006). The degradation of structural elements of EPS enables the detachment of bacteria from location within the biofilm. The interaction between biofilm polysaccharides and extracellular enzymes means the latter is efficiently retained in the biofilm matrix and the polysaccharide can easily be utilised by the attached enzyme. By remaining close to the cell, diffusion distances are short so uptake by the bacteria is rapid if required. Such an interaction has been shown to promote the resistance of these enzymes to proteolysis and augment their thermostability (Skillman *et al.*, 1998). It has recently been shown that a specific PTS is expressed in *S. mutans* biofilms fed with sucrose and that this PTS may be involved in the transport and metabolism of carbohydrates synthesised by GtfB and GtfC (Ajdic and Chen, 2013).

It has importantly been found that concentrations of inorganic ions specifically calcium, phosphate and fluoride are low in dental plaque exposed to sucrose (Cury *et al.*, 2000; Ribeiro *et al.*, 2005). It is likely that this is related to EPS content within the biofilm matrix and this could be an additional factor that contributes to the

cariogenicity of sucrose. Understanding of this aspect is still in its infancy and the associated mechanisms and their impact on augmenting cariogenicity remain to be fully elucidated. The concentrations of calcium and phosphate in oral biofilm are critical in caries development and this is discussed in detail in Section 1.4.5. Importantly, an inverse relationship between caries incidence and the concentrations of these ions in plaque fluid (Margolis and Moreno, 1992) and the plaque matrix (Ashley and Wilson, 1977a) have been revealed.

1.4.3.5 Acid tolerance, acid disposal and alkali generation

There is ample evidence that supports acid tolerance as a trait of crucial significance to cariogenic bacteria (van Houte and Russo, 1986; Marsh, 1989). Acid tolerance infers growth, as well as the survival, of bacterial cells in an acid milieu (van Houte, 1994) and in this way plays a critical role in the expression of virulence of biofilm bacteria and in the pathogenesis of dental caries (Quivey *et al.*, 2000). Following the ingestion of dietary sugars, pH values of lower than 4 are frequently recorded (Stephan, 1944). Few oral bacteria are able to tolerate low pH but some have adopted mechanisms to alleviate the inimical influences of an acidic environment. In particular, mutans streptococci and lactobacilli have developed effective proton-translocating ATPase, enabling the microorganisms to maintain an intracellular pH 0.5 to 1 unit higher than the extracellular environment (Bowen, 2002). The optimum pH for ATPase is 7.5, 6.0 and 5.0 for *S. sanguis*, *S. mutans* and *L. casei*, respectively (Bender *et al.*, 1986). The amount of ATPase produced by these organisms can be enhanced several-fold if they are cultured at low pH values. *Lactobacillus casei* harbours 50 times more ATPase protein than does *Actinomyces viscosus* (Bender and Marquis, 1987). Fluoride, alone or in combination with weak acids, shows promise as a means to disrupt the acid tolerance displayed by these organisms (Eisenberg and Marquis, 1980; Belli *et al.*, 1995); a concept discussed in more detail later in this

thesis (Section 1.5). Controlling caries in this way fails to disrupt the oral microbiota per se but results in a less-acid-tolerant microbiota with reduced virulence (Bowen, 2002).

The disposal of acids and alkali generation play an essential role in plaque physiology yet interest in these phenomena has been sporadic. Many oral microorganisms are found in relatively large amounts in dental plaque despite having poor acid tolerance (Marquis *et al.*, 1987), suggesting they have developed methods to overcome acidic environmental pressures in order to survive. A highly effective mode of proton removal from plaque involves the incorporation of two protons from lactic acid into a proline ring to form δ-amino valeric acid (Stickland, 1934; Curtis *et al.*, 1983). This amino acid was found to be abundant in dental plaque and the major microorganisms involved in this reaction appear to be peptostreptococci (Curtis and Eastoe, 1978). Many microorganisms lacking in acid tolerance mechanisms survive according to the arginine deiminase system; a pathway that involves the generation of ammonia and carbon dioxide to prevent the pH of the immediate environment from dropping too low (Marquis *et al.*, 1987; Curran *et al.*, 1995; Curran *et al.*, 1998). Though this pathway is inducible by low pH, it is suppressed wherever glucose is present. Finally, a major source of alkali in the mouth is the degradation of urea which is constantly present in small amounts within saliva (Kleinberg, 1967) and many microorganisms including *S. salivarius* and actinomyces produce urease (Sissons *et al.*, 1988). In fact, urease activity in *S. salivarius* biofilms was enriched by as much as 130-fold in comparison to fluid chemostat cultures grown in similar conditions (Li *et al.*, 2000). An inverse relationship was demonstrated between the ureolytic capacity of dental plaque and cariogenicity (Clancy *et al.*, 2000).

1.4.4 Current Perspectives in Caries Aetiology

In current times there have been two major schools of thought regarding the role of plaque bacteria in the aetiology of caries. No single microorganism satisfies Koch's postulates to be considered responsible for the condition and dental caries does not fulfil the classical principles of a specific infectious disease (Fejerskov and Nyvad, 2003). The "specific plaque hypothesis" theorises that a small subset of species, namely mutans streptococci, from within the larger context of the diverse microflora of plaque are actively involved in the disease (Loesche, 1986; Tanzer, 1989; Loesche, 1992). This reductionist model is attractive since it implies that a single species or specific number of species could be targeted by preventative measures or monitored to predict infection. However, the theory struggles to account for instances wherein disease occurs in the apparent absence of these putative microorganisms. Therefore, the "non-specific plaque hypothesis" (Theilade, 1986) builds on this and considers that caries is an outcome of the overall activity of the plaque microflora on a whole. Variations on the "ecological plaque hypothesis" reconcile the two earlier hypotheses; particularly recognising the importance of shifts within the proportions of mixed plaque populations while proposing that microorganisms associated with disease may be present at areas that remain sound but not at clinically relevant levels. It suggests that disease, in fact, is the result of a shift in the behaviour of biofilm bacteria in response to environmental changes such as exposure to substrates (Marsh, 1994; van Houte, 1994; Kleinberg, 2002; Marsh, 2003a; Beighton, 2005; Takahashi and Nyvad, 2008) and that the mix of bacteria that emerge are most suited to respond effectively to the niche provided by the ecological change. For instance sugar intake causes plaque pH decline and frequent consumption favours the growth of the acid-producing and acid-tolerating species associated with caries. A dynamic model explains the changes required in the ecology of dental plaque for the development of caries lesions or their remineralisation to occur (Figure 1.4) (Marsh, 1994).

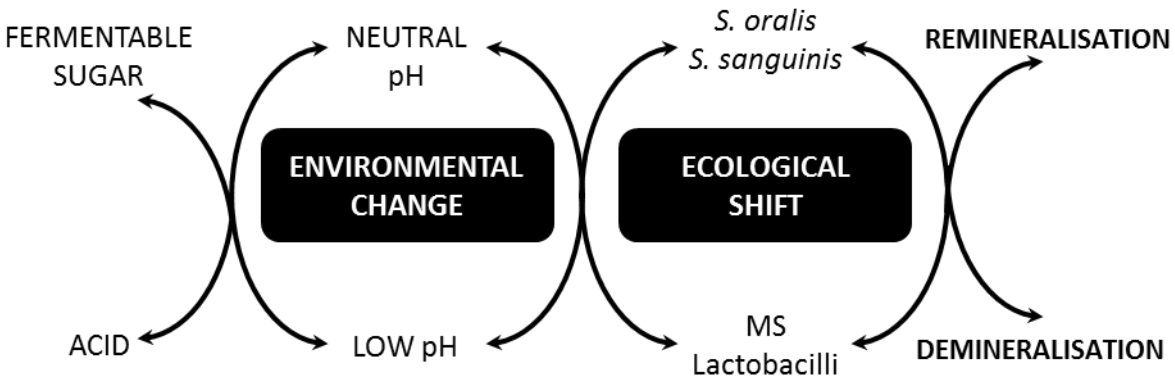


Figure 1.4. The ecological plaque hypothesis in caries etiology. "MS" denotes mutans streptococci. Adapted from Marsh, 1994.

1.4.5 The Chemistry of Enamel Caries

Chemically speaking, enamel caries is a complex process. General principles regarding the kinetics of tooth dissolution are becoming clear and the roles of minor constituents of the enamel mineral are especially crucial; exemplified by the removal of magnesium and carbonate and the uptake of fluoride both resulting in the reduced net loss of mineral (Robinson *et al.*, 2000). Enamel caries is the net outcome of consecutive cycles of de- and remineralisation of enamel at the interface between the biofilm and the tooth surface, when demineralisation outweighs overall remineralisation. An understanding of the biochemical properties of dental tissues is fundamental in understanding their acid dissolution.

1.4.5.1 The microstructure of enamel and hydroxyapatite crystals

Dental enamel is a highly mineralised acellular tissue, comprising predominantly carbonated calcium hydroxyapatite crystals (80 – 90% by volume) (Angmar *et al.*, 1963; Robinson *et al.*, 1971; Robinson *et al.*, 1983; Elliott, 1997) as well as fluid and organic, usually proteinaceous, material (the remaining 10 – 20%). The distribution of these components is not homogenous throughout the enamel and differs as a characteristic of specific tooth morphology. The carbonated-apatite crystals are long

and thin, approximately 50 nm wide in cross-section and more than 100 μm long in the C-axis, extending from the dentine towards the enamel surface (Johansen, 1965). They are packed tightly in bundles of approximately 1000 crystals to form enamel prisms, while it has been shown that some individual crystals may run the full thickness of enamel and fuse with adjacent crystals along their length (Daculsi *et al.*, 1984). When viewed in cross-section, the profile of the prisms can vary from circular to resembling a keyhole and most of the hydroxyapatite crystals are arranged with their C-axes parallel to the long axes of the prism. The space between crystals is occupied by water (11% by volume) and organic material (2% by volume). However, deviation of the crystal orientation from this arrangement can be seen at the boundary of each prism, providing an interface between prisms tending towards more intercrystalline space. This is an important feature in the progression of caries as such spaces are likely to offer diffusion pathways within the tissue. The density of the crystal-lattice structure throughout enamel is non-uniform; generally decreasing from the tissue surface toward the dentine, while porosity, fluid, and organic material increase in this direction (Robinson *et al.*, 2000). In specific locations, such as fissure enamel, the porosity, protein and crystal distribution can be quite complex (Robinson *et al.*, 1971; Robinson *et al.*, 1983). The enamel sampled from fissures shows a highly complicated prismatic arrangement and the low mineral and high protein compositions indicate possible poor prismatic packing (Robinson *et al.*, 1983). In these terms, studying enamel caries of these sites therefore poses clear impediments and selecting samples with simpler morphology such as smooth surface enamel may provide a simpler route for the preliminary stages of model development.

In general terms the mineral component of enamel is a substituted calcium hydroxyapatite that can be expressed using the following stoichiometric formula; $Ca_{10}(PO_4)_6(OH)_2$ (Kay, 1964). Understanding its structure is key in appreciating how

enamel tissue behaves when dissolved by acids. The structure of an individual crystal of apatite can be described as a series of hexagonal plates stacked at a 60° rotation in relation to its immediate neighbour. These hexagonal plates are made up of a hexagon of calcium ions (calcium I) that surround a triangle of phosphate ions which in turn surround a triangle of calcium ions (calcium II) rotated out of phase by 60°. These layers of ions are arranged to enclose a single hydroxyl ion and this central hydroxyl column extends in the c-axis direction through the long axes of each crystal. In reality, the structure of apatite in enamel has been found to vary widely and the degree of variation in enamel dissolution seen between individuals and different tissues can be partially attributed to this variation. Defects and substitutions involving missing ions, particularly calcium (Posner and Perloff, 1957; Winand *et al.*, 1961) and hydroxyl groups (Myrberg, 1968; Young and Spooner, 1969) indeed have a profound effect on the behaviour of apatite under acid dissolution due mainly to their effects on its solubility. For example, the solubility product for enamel mineral (ranging from 7.2×10^{-53} to 6.4×10^{-58}) (Patel and Brown, 1975) is much higher (more soluble) than for stoichiometric apatite; calculated as 3.04×10^{-59} (McDowell *et al.*, 1977). Such variable solubility in enamel could contribute to variations in caries experience amongst individuals and to subsurface lesion formation.

1.4.5.2 Crystal dissolution and growth

Dental plaque, the fluid found in the spaces of the mineral lattice, and oral fluids contain calcium and phosphate ions and whether the environment of the tooth is saturated, under- or supersaturated with respect to these ions is pH dependent. Dissolution or repair of the crystal lattice occurs when the environment is under- and supersaturated respectively. Demineralisation of enamel is initiated when the pH of overlying dental plaque decreases to below critical pH (5.5) though this can differ dependent on the individual. Upon being restored to over pH 5.5, enamel remineralises. Impurities in the tooth mineral such as magnesium, sodium and

carbonate are preferentially dissolved (Robinson, 2009), washed out and not incorporated again during this remineralisation. In this way, balanced cycles of de- and remineralisation result in mineral of better quality; yet if remineralisation cannot counter-balance demineralisation sufficiently, caries lesions develop.

Dissolution of enamel in pure water is relatively fast at first, but slows as dispersed ions that constitute the crystals accumulate in solution. The driving force for enamel dissolution, be it in water or augmented by acids, is the degree of undersaturation of its surrounding solutions. The degree of saturation of a solution can be determined from the solubility product principle which states that the velocity of a reaction is proportional to the product of the masses of the reacting substances, each raised to a power equal to the number of molecules involved (ten Cate *et al.*, 2008). Typically, five calcium ions, three trivalent phosphate ions and one hydroxyl group are released into solution when one unit of solid hydroxyapatite is dissolved (see Eqn 1.1). Once hydroxyapatite containing solutions are saturated and the mineral is in equilibrium with the ions present in solution, the ion activity product of hydroxyapatite (IAP_{HA}) equals the solubility product of this mineral (KSP_{HA}); a constant to the value of 7.41×10^{-60} mol^9/l^9 at 37°C. The solution becomes saturated when phosphate and hydroxyl ions accumulate within solution along with calcium ions. Unlike other minerals, at lower temperatures hydroxyapatite, and therefore enamel surfaces, are more liable to dissolve. Upon the addition of acids, PO_4^{3-} and OH^- ions combine with H^+ ions from the acid to form HPO_4^{2-} ions and water molecules respectively. Ultimately, by removing phosphate and hydroxyl ions from solution the IAP is decreased leading to a solution that is undersatured. The further dissolution of hydroxyapatite then occurs to re-establish the solution as saturated once more.

$$Ca_5(PO_4)_3OH \leftrightarrow 5\ Ca^{2+} + 3\ PO_4^{3-} + OH^- \qquad \text{(Eqn 1.1)}$$

On the other hand, crystals constantly in contact with large volumes of solution saturated with mineral are inclined towards repair. Under the correct conditions new crystals precipitate from solution and small crystals can grow. Growth occurs once the solution's IAP_{HA} exceeds its KSP_{HA}, in other words, once it is supersaturated with respect to hydroxyapatite. Supersaturation can be achieved either by the addition of constituent ions to a solution (e.g. separate soluble salts of calcium and phosphate) or by raising the pH (i.e. by raising the concentrations of PO_4^{3-} and OH^-), or even both. Inherently unstable, supersaturated solutions will propagate the precipitation of new crystals at high enough levels of supersaturation. To balance this, the concentration of ions in solution inevitably drops until it is, once more, merely saturated and remineralisation ceases.

1.4.5.3 Relation of demineralisation and remineralisation to plaque acid-base metabolism

The cycling between the production of acids and bases by plaque bacteria stimulates the demineralisation-remineralisation cycling among tooth, plaque and saliva with plaque as the intermediary component. The role of dental plaque is complex; involving plaque fluid, plaque calcium phosphate, non-complexed plaque calcium and phosphate ions, and plaque pH against the backdrop of tooth calcium phosphate, saliva's ability to deposit plaque calcium phosphate (Ashley and Wilson, 1977b, a), non-complexed saliva calcium and phosphate ions, saliva pH, and plaque acid-base metabolism (Kleinberg, 2002). Demineralisation and remineralisation are likely occurring at the same time within caries lesions (Robinson *et al.*, 2000) and whether a caries lesion develops is largely driven by the frequency and duration of plaque pH cycling between periods of acidic and periods of alkali conditions as well as periods wherein the plaque and tooth mineral are replenished by saliva (Kirkham *et al.*, 1994).

1.5 FLUORIDE IN THE CONTROL OF CARIES

Fluoride is the main active ingredient in currently recommended oral hygiene procedures and is recognised as responsible for the significant decline in caries observed worldwide (Bratthall *et al.*, 1996). This effect can arguably be attributed to a multi-modal programme of treatment including community based interventions (such as water, salt and milk fluoridation); self-applied fluoride (toothpastes, tablets, rinses, gels and foams) and professionally administered fluoride products (such as high fluoride gels and varnishes with recent interest in slow-release technology mounting). Strong evidence suggests that the primary mode of action is the result of its topical activity in the oral cavity (Ogaard *et al.*, 1988; Featherstone *et al.*, 1990; ten Cate, 1990). This thesis focuses on evidence for the chemical interactions of fluoride with components of the oral environment and its resultant anti-caries capabilities.

Oral care products provide a range of benefits that have become an integral part of the daily hygiene routine. They are most commonly applied twice daily and the current understanding of cariostatic mechanisms suggests the regular delivery of fluoride at moderate doses is likely its best method of use. Ideally fluoride should be present at low concentrations in the mouth at all times and to achieve this, therapies should rely as little as possible on patient compliance and remain affordable. Toothbrushing combines the application of fluoride with the removal of dental plaque, contributing not only to the prevention of caries but to preventing periodontal diseases also. It has been well established that the primary control of caries lesion development by fluoride is by its topical effect on de- and remineralisation at the interface of the tooth and oral fluids (Featherstone *et al.*, 1990; ten Cate, 1990) but its role in effecting biofilm bacteria has also been demonstrated. The processes involved in the dissolution and remineralisation of dental enamel have been discussed within this thesis already (Section 1.4.6). Here fluoride's effect on enamel solubility and its

role in favouring remineralisation will be discussed along with its effect on acid production by oral biofilm bacteria and the nature of fluoride inhibition. The concept of plaque as a reservoir for fluoride will also be addressed.

1.5.1 Effect of Fluoride on Enamel Solubility

When in the presence of fluoride, hydroxyapatite behaves as fluorapatite so is afforded a lower dissolving pH of 4.5 – 5.0. Importantly this means that once incorporated into the crystal lattice the enamel, on a whole, is less soluble. When bacteria produce acids, fluoride in plaque fluid penetrates in association with these acids into the enamel subsurface, adsorbs to the crystal surface and protects them from dissolution (Featherstone, 1999). If loosely bound fluoride covers the entire crystal surface it will not dissolve when pH falls due to bacterial derived acids since it now behaves as fluorapatite. If the coating of loosely bound fluoride is partial the uncoated portions of the crystal dissolve (Arends and Christoffersen, 1990). The higher the concentration of fluoride in plaque fluid, the higher the probability it will adsorb to form loosely bound fluoride coatings to protect the crystals. Crucially, very low concentrations of fluoride (sub-ppm range) in solution are already able to substantially inhibit the dissolution of tooth minerals by acids (Featherstone *et al.*, 1990; ten Cate and Featherstone, 1991). It was shown that fluoride concentrations as low as 0.05 ppm (Manly and Harrington, 1959; ten Cate and Duijsters, 1983; Featherstone *et al.*, 1990) present in an acid solution can reduce the solubility of carbonated hydroxyapatite to that equivalent to hydroxyapatite and that higher concentrations of fluoride in solution decrease enamel solubility in a logarithmic pattern (Featherstone *et al.*, 1990).

Fluorapatite forms in the presence of acids, when the concentration of fluoride in solution is low, usually 50 ppm (Eqn 1.2). When the solution bathing the enamel

surface is above 100 ppm ionic fluoride can be taken up with the formation of calcium fluoride (see Eqn 1.3, an example reaction involving a sodium fluoride containing treatment).

$$Ca_{10}(PO_4)_6(OH)_2 + F^- + H^+ \rightarrow Ca_{10}(PO_4)_6(OH)F + H_2O \quad \text{(Eqn 1.2)}$$

$$Ca_{10}(PO4)_6(OH)_2 + 20\ F^- + 8\ H^+ \rightarrow 10\ CaF_2 + 6\ HPO_4^{2-} + 2\ H_2O \quad \text{(Eqn 1.3)}$$

Calcium fluoride forms as spherical globules consisting of poorly crystalline calcium fluoride mixed with phosphate, scattered at the enamel surface. Scanning electron microscopic studies have elucidated that its structure involves mineral growth radiating from a central point of origin (Petzold, 2001). Its formation reaction is concentration and pH dependent so the higher the fluoride concentration the more calcium fluoride is formed. At low pH the solubility of enamel increases and its dissolution provides sufficient calcium for significant calcium fluoride formation.

It is important to note that not all calcium fluoride formed is aggregated at enamel surfaces but also as precipitates within plaque (see Section 1.5.5), in pellicle, in enamel porosities and other inaccessible, stagnation sites. It is known that in such areas the concentration of free ionic calcium is relatively high, meaning it is likely that considerable formation occurs at these sites. In the mouth calcium fluoride habitually dissolves, since oral fluids are undersaturated with respect to this salt. In this way precipitated calcium fluoride can be thought of as a temporary storage of fluoride allowing the gradual release of the active ion. Dissolution rates vary with complete dissolution observed within a day, in exposed surfaces; to days and weeks, in

the rough surfaces of caries lesions and in dental plaque (Brudevold, 1974; Lambrou *et al.*, 1981). After topical treatment with 2% (905 ppm / 47.6 mM F^-) sodium fluoride solution most calcium fluoride deposited on the surface of enamel was dissolved within an hour with levels returning close to baseline by the twenty-fourth hour (Brudevold, 1974). Salivary fluoride concentrations after treatment with 2% (905 ppm / 47.6 mM F^-) sodium fluoride have also been observed due to the dissolution of calcium fluoride in normal conditions. The fluoride concentration remained high for a period after topical application but dropped considerably within 24 hours before returning to normal within a few days (Lambrou *et al.*, 1981).

It is generally considered that the outermost surface of tooth enamel is more resistant to demineralisation than further into the tooth. Marked demineralisation occurs in the subsurface of enamel whilst the surface is only slightly affected when looking at microradiographs of early enamel caries. Without fluoride, demineralisation creates an erosion-like defect, but with fluoride a subsurface lesion is formed (ten Cate *et al.*, 1998). The fluoride concentration in the surrounding fluid determines the mineral content in the surface as revealed by mineral content depth profiles of lesions formed at pH 4.0 (ten Cate *et al.*, 1998).

1.5.2 Role of Fluoride in Favouring Remineralisation

The promotion of remineralisation is essentially a result of the fact that fluoride preferentially replaces OH^- ions within the spaces of the hydroxyapatite crystal lattice since it is a better fit. The suggestion that saliva could remineralise enamel was made as early as 1912 (Head, 1912), explored further in the arrest or disappearance of white spot lesions in 8-year old children (Backer Dirks, 1966) and demonstrated in experimental caries in human subjects (von der Fehr *et al.*, 1970; Edgar, 1978; Edgar *et al.*, 1978). Salivary flow continually buffers the acids produced by biofilm bacteria and as pH returns to higher than 5.5, remineralisation follows. Remineralisation

occurs since saliva is supersaturated with respect to dental mineral. With the presence of traces of fluoride during hydroxyapatite dissolution the solution becomes highly supersaturated with respect to fluorapatite; speeding up the remineralisation process. Fluoride attracts calcium ions to the surface of partially demineralised crystals by adsorbing at these sites (ten Cate *et al.*, 2008). By being less soluble, carbonate-free and low-carbonate apatite form preferentially to original mineral via the nucleating action of partially dissolved minerals. Under the influence of fluoride, this process is aided. Over the course of repeated dissolution and deposition events, enamel crystals transform and may have completely different characteristics compared to their original state (Featherstone, 1999; ten Cate *et al.*, 2008). Due to the exclusion of carbonate and incorporation of fluoride, the resulting enamel is more resistant to future acid challenges.

1.5.3 Effect of Fluoride: Microbial – Biofilm Aspects

The effects of fluoride on oral microflora have been studied extensively, yet there is little consensus that its antimicrobial effects and influence on bacterial acid production contribute to the overall anti-caries effect of fluoride to a significant degree (Hamilton, 1990; ten Cate, 1999). A multitude of direct and indirect effects on bacterial cells have been documented (Hamilton and Bowden, 1996; Marquis *et al.*, 2003) yet the opinion held by many is that it is likely they are unimportant as a caries-preventive action of fluoride (van der Hoeven and Franken, 1984). The concentrations needed for the inhibition of the metabolism of oral microorganisms is much higher than is required to influence the de- and remineralisation processes. Therefore, in reality the effect on enamel solubility and promotion of remineralisation is considered to be the principal mode that fluoride achieves its cariostatic and anti-caries effects (Geddes and Bowen, 1990). The role of fluoride in the metabolic activity of plaque bacteria has been questioned since it was shown that there are no

differences in the mutans streptococci populations in children of fluoridated and non-fluoridated communities and only large concentrations of fluoride seem to be effective (Burt *et al.*, 1986). While there is evidence that fluoride, even at low concentrations, can affect bacterial metabolism (Marquis, 1990) it was recently shown that the use of 1,000 ppm (52.6 mM F^-) fluoride toothpastes generated low levels of plaque and salivary fluoride which were in fact insufficient in exerting a significant antimicrobial effect on plaque bacteria (Lynch *et al.*, 2004). Despite this, small but perceptible antimicrobial effects have been reported when fluoride is present extracellularly as low as 53 μM F^- (10.07 ppm) (Bibby and van Kesteren, 1940; Kashket *et al.*, 1977), and when the intracellular fluoride levels can be near 320 μM F^- (60.8 ppm) (Kashket and Preman, 1985). It may be misconceived that these small antibacterial effects pose no consequences in the reality of the complex community of dental plaque. Nonetheless, its effects on the rate of acid formation *in vivo* should be considered seriously as a more significant factor than the pH minimum that is achieved in the presence of fluoride; since it would continually influence the diffusion and buffering of acids in the ecosystem of the plaque. Also, fluoride inhibits microorganisms more effectively as plaque pH falls (as is the case following food consumption) even if present at the same concentration; a crucial action. Furthermore, the role of fluoride counter ions commonly present in toothpastes such as amines ($C_{27}H_{60}F_2N_2O_3$), stannuous (SnF_2), and sodium (NaF) as well as the effect of preservatives, surfactants and antimicrobials such as zinc salts, triclosan and essential oil extracts seems important in the possible mechanisms of plaque control (Marsh, 2012). Though the *in vivo* implications remain unclear, it has recently been highlighted that the concentrations of fluoride found in dental plaque have proven biological effects on virulence factors of *S. mutans in vitro*, such as glucan synthesis and the production of acids (Koo, 2008). Nonetheless, an understanding of the effects of fluoride on the behaviour of oral bacteria is important for the aims of the work presented in this thesis, so a brief review is included here. Our understanding of the effect of fluoride on the synthesis

of extracellular polymers and on the production of acids is presented together with evidence of the antibacterial effects of topical fluoride and its influence on the formation and bacterial composition of dental plaque.

1.5.3.1 Effect of fluoride on intra- and extracellular polymer synthesis

IPS and EPS are key features in the cariogenicity of dental plaque bacteria (Section 1.4.3.4). Streptococci, lactobacilli and actinomyces in particular form glycogen as their principal intracellular carbohydrate store for degradation when exogenous nutrient levels are low (Hamilton, 1976; Hamada and Slade, 1980). It was revealed in early reports that low concentrations of fluoride affected glycogen formation (Weiss *et al.*, 1965; Hamilton, 1969; Sandham and Kleinberg, 1969). It was later shown that high concentrations of fluoride did not inhibit enzymes of the glycogen synthetic pathway nor the degradative enzymes, glycogen phosphorylase (Kanapka and Hamilton, 1971). Inhibition of glycogen formation has therefore been attributed to indirect processes, such as the unavailability of ATP and glucose-6-P required for glycogen to be synthesised. Plaque fluoride inhibits the synthesis of glycogen intracellularly since the process is dependent on the fluoride-sensitive uptake of sugars by the bacteria (Wegman *et al.*, 1984). Dental plaque EPS from subjects residing in areas where water fluoridation was low (0.2 ppm/10.52 μM F^-) was higher, 15.2%, than from those exposed to higher fluoridated water (1.0 ppm/52.6 μM F^-), 10.3% and fluoride was only shown to exert an effect on EPS levels at 10 ppm F^- *in vitro* (Broukal and Zajicek, 1974). However, this work has not been confirmed in other studies like it. Some report that the synthesis of EPS is increased dramatically in the presence of fluoride, for instance in mutans streptococci in the presence of sodium fluoride or stannuous fluoride and in non-mutans streptococci, though, only by stannuous fluoride (Zameck and Tinanoff, 1987). It was recently shown, *in vitro*, that EPS formation was altered when *S. mutans* biofilms were exposed to a range of

concentrations of sodium fluoride (0, 1, 3, 10, 30, 100, 300, 1,000 and 2,000 ppm – 0, 52.6 μM, 158 μM, 526 μM, 1.58 mM, 5.26 mM, 15.8 mM, 52.6 mM and 105 mM F^- respectively) (Pandit *et al.*, 2013). However, no effect was observed on GTF activity (Pandit *et al.*, 2013). Both biovolume and thickness of EPS were affected in a sigmoidal pattern in response to this range of concentrations, suggesting fluoride has minimum and maximum effective concentrations against this virulence factor. Thickness was reduced with fluoride at concentrations from 30 ppm upwards (1.58 mM) with maximum effect on this and biovolume determined to be at ≥ 100 ppm (5.26 mM) F^-. The disparity between the unaffected GTF activity and the observed effect on EPS, suggests fluoride's mechanism in reducing EPS synthesis may be due to its activity in disrupting proton permeability and the F-ATPase activity of biofilm cells in reducing the secretion of GTFs even if they are formed. Other research also confirmed that GTF and FTF are indeed not sensitive to fluoride (Sharma *et al.*, 1974; Schachtele *et al.*, 1977). Treasure (1981) reported increased EPS was formed when *S. mutans* and *A. viscosus* were exposed to 5.3 mM (100.7 ppm F^-) fluoride and that the proportions of water soluble and glucan polymers rose too; whilst the synthesis of water-soluble fractions was unaffected and the formation of insoluble polymer was significantly inhibited in another study (Shimura and Onisi, 1978). Bowen and Hewitt (1974) showed that *S. mutans* in the presence of 3.7 mM (70.3 ppm F^-) fluoride and sucrose formed extracellular polysaccharides with an increase in the ratio of fructose to glucose. The dose dependent reduction in EPS thickness shown in Pandit's study (Pandit *et al.*, 2013) reveals that high concentrations of fluoride may be capable of penetrating further into biofilms than at lower concentrations. This is supported by evidence that biofilms treated with 1,000 ppm (52.6 mM) F^- for 30 minutes maintained biofilm fluoride levels and that the fluoride could penetrate at this concentration (Watson *et al.*, 2005).

1.5.3.2 Effect on bacterial acid production

Utmost interest in caries prevention research has been in studying the effect of fluoride in the inhibition of acid formation. Studies have focused primarily on *S. mutans* from plaque in humans and animals, salivary sediment and pure cultures of other oral bacteria (Hamilton, 1990). Evidence indicates that the fluoride sensitivity of various genera and species is markedly varied and influenced by the acidic conditions of the plaque environment. *S. mutans*, for example, was found to be 20- to 40- times more sensitive to sodium fluoride than *Lactobacillus casei* and 7- times more sensitive than some species of *Actinomyces* (Hamilton and Bowden, 1988). Early work showed that 1 – 2 ppm (52.6 – 105.2 μM) fluoride had a detectable effect on acid production but 10 ppm (526 μM) were needed for decisive inhibition in pure cultures of various oral acidogenic bacteria, (Bibby and van Kesteren, 1940; Murray *et al.*, 1991) and in mixed cultures of salivary organisms (Wright and Jenkins, 1954). It was also found that the sensitivity of bacteria to fluoride greatly increased as pH fell (Shiota, 1956; Jenkins, 1959). Acid production was completely inhibited when saliva adjusted to pH 5.0 was incubated with sugar and fluoride solutions of concentrations of 6 ppm (316 μM) and over. In fact pH rose due to the continued unopposed bacterial production of bases (Jenkins, 1959).

The findings of some *in vitro* work have provided clear evidence for the influence of topical fluoride, not only on reducing enamel solubility but also on acid production. For instance, the protection against demineralisation of fluoride treated (as acidulated phosphate fluoride; APF) enamel was much greater in test groups exposed to 2% (58.40 mM) sucrose fed *S. mutans* biofilm than in those treated with lactic acid (Zahradnik *et al.*, 1978). The inhibition of demineralisation was mediated by the deposition of calcium fluoride on the enamel using KOH, while SEM revealed that less *S. mutans* were attached to the surfaces when treated with this fluoride source.

Additionally, the daily use of a fluoride rinse lead to reduced plaque acidogenicity in the presence of sucrose and increased plaque fluoride concentration but this anti-caries effect was lost once daily rinsing was ceased (Geddes and McNee, 1982) whereas acid production fell in xerostomic patients using fluoride containing gel over a 5 year period (Brown *et al.*, 1981). Woolley and Rickles (1971) were able to show that the topical application of fluoride must be frequent to have a significant effect on acid production. In their study, the inhibitory effect on acid production of 2% (47.6 mM F^-) sodium fluoride solutions applied daily for 1 week was highly significant at 8 hours but not after 3 – 4 days or 1 week. Moreover, it was shown in a recent *in vitro* study that systems involved in acid production and acid tolerance in biofilms of *S. mutans* appear to be more susceptible to fluoride than systems of EPS synthesis (Pandit *et al.*, 2013). The authors demonstrated that fluoride concentration was closely related to its activity in affecting acid production and acid tolerance in these biofilms and that the relationship also followed a sigmoidal pattern. Here a reduction in the initial proton production levels was observed when biofilm was exposed to concentrations from 10 ppm (0.526 mM) F^- with maximum efficacy at concentrations including and over 30 ppm (15.8 mM) F^-. Increased proton permeability was elucidated from 10 ppm F^- with maximum efficacy at concentrations including and over 300 ppm (15.8 mM) F^-. Reductions in acid tolerance were demonstrated when fluoride was able to increase the final biofilm pH values; the final concentrations of protons decreased from as low as 1 ppm (52.6 μM) F^- but did not decrease further at concentrations of 30 ppm F^- and above. The activity of F-ATPase; a key determinant of acid tolerance (Lemos and Burne, 2008) was also affected when concentrations of fluoride were high; at 100 and 300 ppm F^- (Pandit *et al.*, 2013). Potassium fluoride solutions (0 – 225 ppm i.e. up to 11.8 mM F^-) added to suspensions of *A. naeslundii*, *A. oris*, *S. mutans* and *S. sanguinis* reduced acid production from 10 mM glucose in all bacteria dose-dependently (Kawashima *et al.*, 2013). However, it was evident that, *Actinomyces* species were more tolerant to fluoride than *Streptococcus*. The

production of acids almost ceased in the *Streptococcus* groups when incubated at pH 7.0 with 90 ppm (4.74 mM) F^- while *Actinomyces* acid production remained continuous and even higher in comparison. *Actinomyces* were also more tolerant to fluoride than *Streptococcus* when pH was lowered to 5.5. Without fluoride the acidic end-products from *A. oris* were 80% lactate, with minor proportions of acetate and formate. The proportion of lactate was decreased to 70% in the presence of 225 ppm (11.8 mM) F^-. In comparison, an *in vivo* study revealed that when microcosm plaque bacteria were exposed for 10 minutes to 225 and 900 (47.4 mM) ppm F^-, lactate production from 10% (555 mM) glucose was inhibited by 34% and 46% respectively (Takahashi and Washio, 2011). Metabolome analyses elucidated that the *in vivo* effects of fluoride on supragingival plaque sugar metabolism and acid production were consistent with those found *in vitro*; and confirmed that fluoride inhibited bacterial enolase and disrupted the Emden-Meyerhof pathway.

1.5.3.3 Evidence of antibacterial effects of topical fluoride and its impact on plaque formation and bacterial composition

Early work showed that 100 ppm (5.26 mM) fluoride was required to reduce growth in pure cultures of various oral acidogenic bacteria, (Bibby and van Kesteren, 1940) and in mixed cultures of salivary organisms (Wright and Jenkins, 1954). Although cellular membranes (Yotis *et al.*, 1983) and cytoplasmic proteins (Kashket and Bunick, 1978; Katayama *et al.*, 1981) can bind fluoride, significant differences observed in the binding of ^{18}F among a number of oral bacteria indicates clear differences in either the number of F-binding sites or the affinity of the existing sites for fluoride (Yotis and Brennan, 1983). This, and some of the evidence already presented, suggests that fluoride should exert a differential effect on the composition of dental plaque but in reality, while high concentrations of fluoride administered topically may bear selective properties on the microflora of dental plaque (Loesche *et al.*, 1973; Loesche *et al.*, 1975; Brown *et al.*, 1983), naturally occurring fluoride has not

been shown to effect dental plaque composition in this way, even at concentrations as high as 1 mM (19 ppm) F^- (Kilian *et al.*, 1979; Bowden *et al.*, 1982). Despite the early *in vitro* evidence that fluoride at extremely low concentrations can inhibit bacterial growth (Maltz and Emilson, 1982; Milnes *et al.*, 1985), the levels of fluoride achieved *in vivo* are insufficient to inhibit growth, except at the high levels associated with typical applications of topical fluoride.

The effect of a daily application of 1% sodium fluoride (452.5 ppm/23.8 mM) gels on bacterial counts, and on lactic and acetic acid synthesis was tested in plaque from xerostomic patients over a 5 year period (Brown *et al.*, 1981). The deficient saliva flow of these patients meant the clearance of applied fluoride would be delayed so that bacteria were exposed to fluoride for much longer than would occur in subjects with a normal saliva flow. Concentrations of fluoride in the plaque increased while acid production and bacterial counts fell. There is additional evidence of the selective inhibition of *S. mutans* in approximal and occlusal plaques after daily applications of APF gel (Loesche *et al.*, 1973; Loesche *et al.*, 1975) while plaque scores were reduced. It was suggested that since *S. mutans* colonises only plaque or the surface of enamel that if it is suppressed by APF there is no reservoir elsewhere capable of recolonizing the plaque quickly. Whilst organisms like *S. sanguinis* exist on many oral surfaces and can rapidly colonise cleaned enamel (Murray *et al.*, 1991). Further support for the antibacterial effect of topical fluoride was evident when a differential degree of demineralisation was observed in enamel by *S. mutans* biofilm exposed to either fluoride, nigericine or neither (van Loveren *et al.*, 1987). Like fluoride, nigericine reduces the difference between the intracellular and extracellular pH of bacterial cells yet without any effect on the solubility of enamel. In this *in vitro* study, microradiographic profiles were captured and the level of calcium dissolving from the enamel was measured. Of the three test scenarios, a markedly higher level of

protection was observed in samples exposed to fluoride. The amounts of enamel dissolved were inhibited by 89% and 68% in the fluoride and nigericine groups respectively, suggesting that inhibition of acid production may be an underestimated aspect of fluoride's activity. More recently, no effect on bacterial cell viability was observed when *in vitro S. mutans* biofilms were exposed to sodium fluoride solutions up to 2,000 ppm (105 mM) F^- in concentration (Pandit *et al.*, 2013). In this study, biofilms were also unaffected in terms of bacterial biovolume and thickness as visualised by CLSM. However, it provided evidence that fluoride at high concentrations may affect the structural integrity of *S. mutans* biofilm cells. The technique revealed that at 100 and 1,000 ppm (5.26 and 52.6 mM respectively) F^- exposures bacterial mass appeared to be smaller and more scattered than when exposed to 0 or 10 ppm F^- (526 μM; Pandit *et al.*, 2013). Fluoride also inhibited the growth of bacterial suspensions of *A. naeslundii*, *A. oris*, *S. mutans* and *S. sanguinis* in a dose dependent manner though the growth of *Actinomyces* was shown to be more tolerant to the effects of fluoride (Kawashima *et al.*, 2013).

However, Brown (1981) also observed that not all *S. mutans* and *Lactobacillus* spp. were eliminated. An increasing proportion of *S.mutans* with fluoride resistance characteristics was reported; though it is presumed that enough bacterial cells remained susceptible since acid production continued to reduce regardless (Brown *et al.*, 1981). Fluoride-resistant bacteria have been reported previously *in vitro* in oral streptococci (Williams, 1964; Hamilton, 1969) as well as in lactobacilli (Green and Dodd, 1957) and using animal models (van der Hoeven and Franken, 1984). It is known that fluoride resistance is not due to changes in the sensitivity of enolase or to the cell's inability to take up fluoride but the precise mechanism that confers fluoride resistance is so far unknown (Kashket and Preman, 1985). In some species, the resistance is lost when grown in fluoride-free conditions for several generations, but

in others the effect remains after exposure to fluoride ceases and is presumably genetic. It was shown that frequent exposure even to 1,000 ppm (52.6 mM) fluoride had not induced significant resistance when the plaques of users of fluoride toothpaste were studied with this concern in mind (Eisenberg *et al.*, 1985). The dental plaque derived from these individuals was still sensitive to 5 ppm (0.263 mM) F^-. Currently, there are no reports of metabolic adaptation to fluoride by dental plaque microorganisms though it has been suggested that adaptation to fluoride reduces the acidogenicity of cells (Hamilton and Bowden, 1988) since caries progression in xerostomic patients with high levels of cariogenic bacteria was limited by fluoride (Brown *et al.*, 1983). Reductions in acidogenicity could be indicative of cell protection against fluoride since the fluoride sensitivity of cells is potentiated by increased H^+ in the environment (Kashket *et al.*, 1977).

1.5.4 Nature of Fluoride Inhibition

The mechanism that fluoride uses to affect GTF and the subsequent production of EPS, an essential feature in the pathogenesis of caries (Bowen and Hewitt, 1974; Marquis, 1995), has been discussed already (Section 1.5.3.1). Yet the primary site of fluoride inhibition is its inhibition of the enolase enzyme of the Embden – Myerhof pathway of acid production (Borei, 1945) and its influence in undermining sugar uptake (Figure 1.5). The metalloenzyme enolase converts phosphoglyceric acid (PG) to phosphoenolpyruvic acid (PEP) and when blocked, PG accumulates while latter products such as PEP and lactic acid cannot be formed. The addition of 2.4 mM (45.6 ppm F^-) sodium fluoride to glycolysing cells of *S. salivarius* at pH 5.8 caused a rapid increase in PG while the concentration of PEP within the cells dropped (Kanapka and Hamilton, 1971; Hamilton, 1977); demonstrating the fluoride sensitivity of the enolase enzyme. The concentration of ATP and glucose-6-P was also significantly

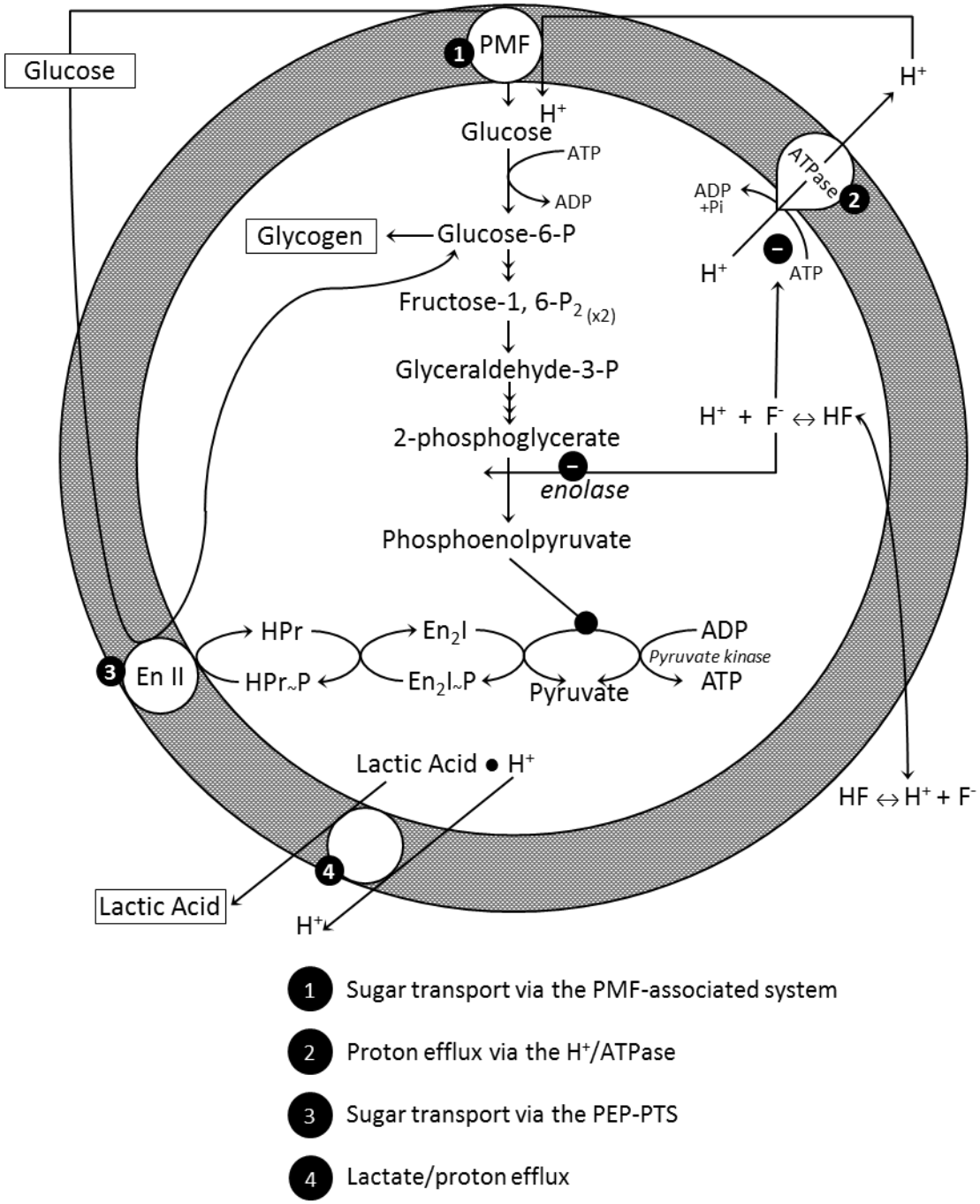


Figure 1.5. Direct and indirect interaction of fluoride with essential reactions involved in carbohydrate metabolism by oral bacteria: Adapted from Hamilton, 1990.

reduced; partially reflecting the decreased conversion of PEP to pyruvate mediated by pyruvate kinase which would fully impair a variety of cellular processes.

Accumulated PG and decreased PEP impact the plaque bacteria in a number of ways (Murray *et al.*, 1991). Foremost, the ability of the biofilm bacteria to cause caries is impaired due to the reduced production of lactate. It has been shown that fluoride interferes with the uptake of glucose by two independent mechanisms; on the one

hand by reducing PEP production and on the other, by inhibiting proton extrusion (Figure 1.5). In most bacteria the uptake of glucose requires the presence of PEP (as part of the PEP-PTS system; see Section 1.4.3.3), so uptake is reduced by fluoride as indicated by the reduced glucose-6-P observed in the above study (Kanapka and Hamilton, 1971; Hamilton, 1977). While some bacteria, including *S. mutans*, take up glucose (and other nutrients) via an additional mechanism; proton motive force (PMF) (Hamilton, 1990). In fact, according to the chemi-osmotic theory, a number of metabolic processes are driven by the potential energy derived from transmembrane proton electrochemical gradients such as this (Mitchell, 1966).

Uptake by this route depends on the ability of the microorganism to expel protons with the expense of ATP (Harold, 1977) as well as by the efflux of end-products (Michels *et al.*, 1979) (Figure1.5; label 2). This is controlled by fluoride-sensitive enzymes; the proton-translocating ATPases which are reportedly inhibited by concentrations of fluoride as low as those in plaque fluid (Psarros *et al.*, 1990). A pH gradient and electrical potential gradient are formed via the expulsion of protons across the cellular membrane and these relate to the proton motive force achieved. By this process, glucose and other solutes can be transported into the cell against concentration gradients in symport with a proton as that proton moves into areas of lower intracellular proton concentration (Figure 1.6). Therefore, transport via this mechanism hinges on the continuous expulsion of protons and complements the PEP-PTS mode of transport in particular environmental conditions (Hamilton, 1987). In fact the ability to maintain pH homeostasis within cells is a useful adaptation of acidogenic and aciduric oral bacteria during growth in acidic environments (Kobayashi, 1987). The expulsion of protons ensures a relatively alkaline intracellular environment suitable for the efficient activity of essential catabolic and biosynthetic enzymes within the bacterial cell. A major factor in the anti-microbial

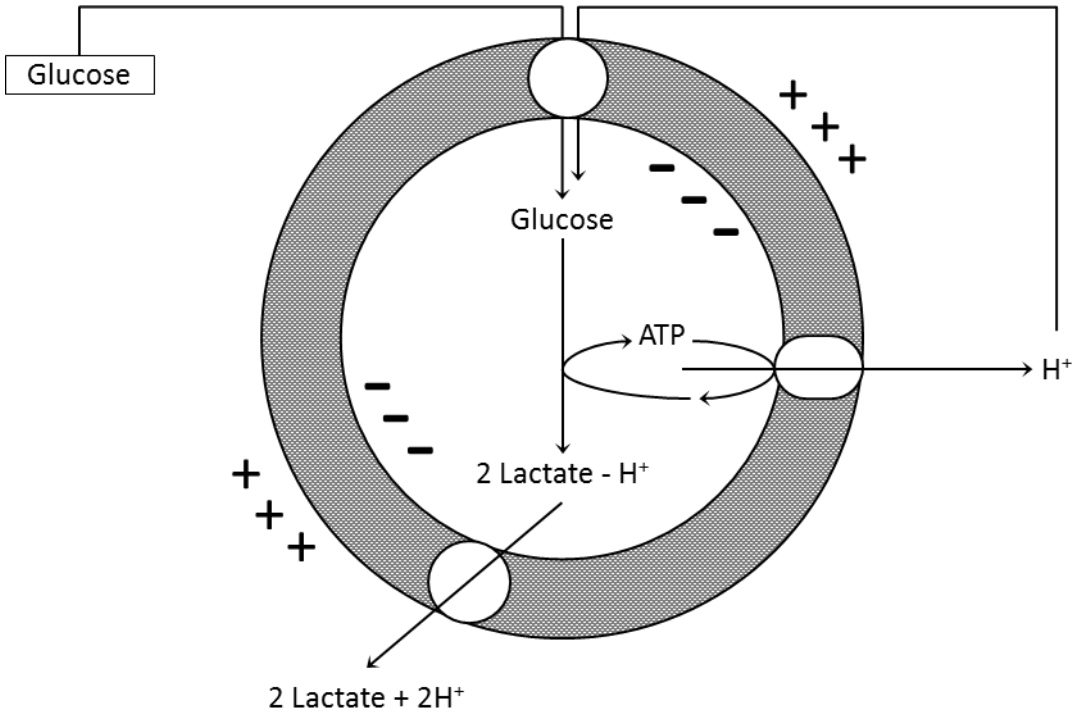


Figure 1.6. Proton gradients in anaerobic oral bacteria by membrane H^+/ATPase activity and lactate effluxand uptake of protons in symport with glucose. Adapted from Hamilton, 1990.

effect of fluoride is its disruption of cellular pH homeostasis and the direct inhibition of proton-pumping ATPases (Marquis, 1977; Sutton *et al.*, 1987); the dissipation of proton gradients influencing proton motive force; and the indirect inhibition of ATP formation via the direct inhibition of enolase. All influence pH homeostasis capabilities.

As illustrated in Figure 1.7, the accumulation of fluoride by oral bacteria was shown to occur when grown in the presence of the ion in proportional amounts to the fluoride level of the external fluid phase reaching levels intracellularly higher than in the growth medium (Jenkins and Edgar, 1969, 1977). Studying *S. sanguinis*, it was revealed that fluoride indeed accumulated without active transport mechanisms that depended on available energy and uptake proceeded against concentration gradients (Kashket and Rodriguez, 1976). Following this, studies of *S. mutans* confirmed the

energy-independent uptake of fluoride and its increase in response to decreases in

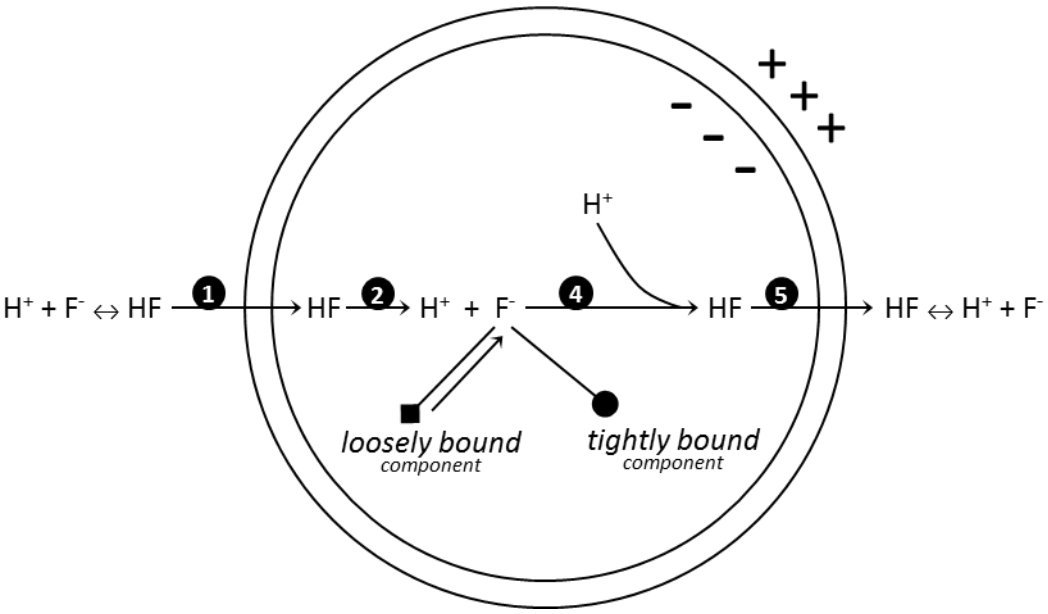


Figure 1.7. The accumulation, intracellular processes and efflux of fluoride in bacterial cells. Adapted from Hamilton, 1990.

external pH. It was concluded that at low pH fluoride behaves as a weak acid hence in the protonated form it can diffuse into cells as HF (Belli *et al.*, 1995).

By attempting to maintain the intracellular pH as near to neutral as possible, bacteria constantly possess a flux of pH gradients in response to their environments so when pH is low more fluoride accumulates in the cell when the difference between extracellular and intracellular pH is large (Borei, 1945; Eisenberg and Marquis, 1980; Vicaretti *et al.*, 1984; Kashket and Preman, 1985). Fluoride-sensitive organisms, including *S. mutans*, generate increasingly larger pH gradients as the pH drops while those resistant to fluoride, such as *Lactobacillus casei*, seem to avoid fluoride inhibition by conserving the pH gradient to as small as possible; unless extracellular pH drops below pH 5 (Hamilton *et al.*, 1985).

At extracellular pH values lower than neutral, and once transported into the cell, fluoride dissociates into H^+ and F^- (Figure 1.7, reaction 2) if the cytoplasm is more alkaline than the extracellular environment. The fluoride component can then either bind loosely or into smaller tightly bound fractions (Figure 1.7) and these directly influence cellular enzymes to elicit the primary inhibitory effect observed. Alongside this, the influx of protons during this process acidifies the cytoplasm and if it continues, the intracellular environment may be too low for the optimum activity of enzymes involved in catabolism and biosynthesis (Hamilton, 1990). By increasing the acquisition of protons and reducing the cell's capacity to extrude protons via ATPases, fluoride, effectively reduces the tolerance of oral bacteria to metabolism and growth in acidic situations (Eisenberg and Marquis, 1980; Bender *et al.*, 1986; Sturr and Marquis, 1992). Additionally, by dispelling the transmembrane pH gradient, the influx of protons influences processes that use the energy of proton electrochemical gradients (Hamilton *et al.*, 1985; Kashket and Kashket, 1985). As a final consequence the reduced glucose uptake prevents the synthesis of the intracellular polysaccharide; glycogen that acts as a store of carbohydrate (Figure 1.5), enabling oral bacteria to continue to produce acids after the dietary sugar has been washed away by saliva (see Section 1.4.3.4).

1.5.5 Plaque as a Fluoride Reservoir

A number of clinical studies support the concept that the amount of fluoride in oral reservoirs is crucial for its anti-caries effect, since an inverse relationship has been demonstrated between caries incidence and the concentration of fluoride in saliva and/or dental plaque (Gaugler and Bruton, 1982; Schamschula *et al.*, 1985; Nobre dos Santos *et al.*, 2002). Maintaining a cariostatic concentration of fluoride in oral fluids is vitally important in increasing the anti-caries effects of fluoride agents (ten Cate, 1997; Featherstone, 2000). This is chiefly achieved by the release of the ion from

bioavailable reservoirs on the teeth, oral mucosa and dental plaque that can persistently increase fluoride concentrations in the fluids in contact with de- and remineralising teeth. This is particularly interesting in the case of dental plaque due to its association with the caries disease process and could be manipulated to achieving heightened anti-caries effects. Considering that most individuals cannot completely remove dental plaque after toothbrushing, the amount of fluoride retained in plaque can help determine the fate of the enamel beneath it (Pessan *et al.*, 2006; Pessan *et al.*, 2008; Pessan *et al.*, 2010; Buzalaf *et al.*, 2011). Since fluoride retained in plaque can be released under acidic conditions during cariogenic challenges, its retention so close to the site of de- and remineralisation is highly clinically relevant. In other words, it means that fluoride is released and made available when it is needed, to reduce demineralisation or to enhance remineralisation of early lesions. Calcium plays a key role in the formation of oral fluoride reservoirs which can either be mineral deposits of fluoride including calcium fluoride (CaF_2) and fluorapatite (FAp); or biologically bound calcium-fluoride (Ca-F) deposits (Vogel, 2011). In fact, fluoride does not easily penetrate plaque, but seems bound within the matrix of this biofilm (Watson *et al.*, 2005). This thesis Section focuses on the significance of the latter; in how bacteria and other components of the dental plaque constitute a source of fluoride.

Plaque fluid is greatly supersaturated with respect to fluorapatite, yet no significant stores of this mineral have been discovered in plaque. This is due to fluorapatite's sensitivity to high concentrations of mineralisation inhibitors in the oral environment (Tomazic *et al.*, 1975; Hay *et al.*, 1979). The insolubility of this mineral appears to negate its value as a source of oral fluid fluoride within plaque. Therefore CaF_2-like deposits and bacterial / biological Ca-F appear to be the major plaque fluoride reservoirs capable of increasing plaque fluid fluoride concentrations (Rolla and

Saxegaard, 1990; Rose *et al.*, 1996; Larsen and Richards, 2001; Ogaard, 2001; Whitford *et al.*, 2002). It has been proposed that fluoride reacts with intercellular or intracellular calcium "bridges" to form calcium-fluoride bonds at fixed anionic bacterial sites (Rose *et al.*, 1996). The theory that fluoride breaks bidentate calcium bonds is important in this model since it leads to more calcium and fluoride binding. It was demonstrated that bacterial Ca-F binding was a continuous function of the concentrations of these ions and dependent on the binding capacity of the bacteria as well as on pH (Rose *et al.*, 1996). The pH dependence arises since calcium and hydrogen ions compete for the same anionic sites on the bacterial surface. Non-bacterial biological fluoride binding includes oral proteins, mucosal tissue and bacterial fragments remaining within the dental plaque. These would appear to possess binding characteristics similar to those of bacteria; also involving the formation of calcium-fluoride bridges (Vogel *et al.*, 2010). Therefore, unless additional calcium is supplied along with conventional topical fluoride containing agents, reservoirs appear to form primarily as bacterial / biological Ca-F deposits.

1.6 Approaches in Studying Dental Caries

The requirement for appropriate laboratory models of dental plaque and the processes of dental caries has been propelled by the search for new anti-caries therapies and by the need for accurate and highly representative studies of the disease process. Key in the criteria for these models is the ability to be predictive of clinical outcomes, the use of orally relevant organisms proven present in oral biofilms, contact time relevant to the use of oral care products and reproducibility. So far we can see that dental caries is a complex, multifactorial phenomena so it must be appreciated that approaches in studying it are diverse and accompanied with inherent strengths and limitations. This section will review a number of these approaches, highlight key findings in their application and discuss some of the considerations required in formulating specific

experimental design. A number of models have been developed and applied to study various aspects of the dental caries disease process but few *in vitro* ones incorporate the complexity of the plaque mediated disease alongside studying changes in the enamel. In addition, a subsequent discussion of some of the common techniques used in assessing enamel demineralisation and biofilm cariogenicity is presented.

1.6.1 Abiotic Model Systems

Abiotic model systems do not model the biological aspect of the dental caries disease process. Instead they focus on one component; the dissolution of enamel by acids, meaning they are inherently reductionist. Work using these approaches has provided invaluable insight into the chemistry and structure of enamel along with the fundamental mechanisms of mineral dissolution and growth by focusing on varying one parameter at a time. Though they fail to take into account all parameters representative of the natural situation, chemical systems are typically easy to operate and reproduce especially in comparison with those that include microbial ecology. Artificial caries lesions may not be identical to natural enamel lesions but it has been shown that they are, in general, similar (Arends and Christoffersen, 1986). Within the field of purely chemical models there are those that model only the demineralisation process and those that take into account remineralisation also.

1.6.1.1 Demineralisation experiments

Some demineralisation experiments have appeared earlier in this thesis, indicating the key role of this approach in the history of cariology. When, in 1819, Parmly theorised that an unidentified "chymical agent" was responsible for caries it led to the observation that dilutions of nitric and sulphuric acid could dissolve enamel and dentine (Robertson, 1835; Regnart, 1938; Newbrun, 1983). Since then numerous lesion-producing acid systems have been used to study the dissolution of enamel

(Silverstone and Johnson, 1971; Moreno and Zahradnik, 1974; Arends and Schuthof, 1980; Arends *et al.*, 1980; Clarkson *et al.*, 1981). Both charged (Silverstone and Johnson, 1971) and uncharged acidified gels (Groeneveld and Arends, 1975) have been used to form subsurface lesions like those found in natural caries. Later it was shown that gels are not necessary for surface layer formation and that an acidic, undersaturated calcium phosphate solution formed lesions suitably (Larsen, 1974; Moreno and Zahradnik, 1974). In agreement with *in vivo* observations (Arends *et al.*, 1984; Ogaard *et al.*, 1986a; Ogaard *et al.*, 1986b) that pellicle formation was not the cause of subsurface demineralisation, simple chemical models were able to show convincingly that subsurface lesion formation is possible without pellicle formation *in vitro* (ten Cate and Duijsters, 1983; Borsboom *et al.*, 1985).

1.6.1.2 pH cycling models

pH cycling models mimic the pH changes that occur in the mouth; typically they begin with forming a lesion with an appropriate demineralising solution then subjecting specimens to a period of pH cycling. Early pH cycling models were relatively simple, using purely inorganic solutions to give rise to the dissolution or deposition of mineral in the enamel (ten Cate and Duijsters, 1982). This was improved upon by taking into account findings observed *in vivo* so that the resulting model was a step more representative (Featherstone *et al.*, 1986) though it produced caries on a quicker scale than is typically seen in nature. White (1987) too incorporated an inorganic demineralisation challenge yet followed it with a remineralisation phase using saliva. Important for predicting *in vivo* phenomena this *in vitro* model means that the biochemical processes of salivary enzymes were taken into account. In these models, samples can be analysed during the cycling period, primarily by analysing the solutions in which they are situated, to give an indication of the ongoing effects of an agent for instance. On the other hand, direct assessment of the mineral content

profile within the enamel lesions can be obtained by analysing samples upon completion of the cycling experiment.

1.6.2 Bacterial Model Systems

When investigating phenomena involving microbial ecology, such as biofilm-mediated diseases, the possible approaches to employ form a hierarchy wherein the most representative are studies of the natural ecosystem itself. *In situ* investigations achieve this but face some obstacles with respect to cost, reproducibility and the requirement of ethical approval. The experimental model system constitutes the polar opposite scenario. They seldom attempt to include all aspects of the complexity of the natural system. With the hope of finding answers to clear questions posed, experimental models are often simplified for the investigation of a small number of parameters and superfluous components are generally held constant or simply ignored. Bridging the gap is the microcosm which can be defined as "…an attempt to bring an intact minimally disturbed piece of an ecosystem into the laboratory for study in its natural state" (Pritchard and Bourquin, 1984). By aiming to correspond directly to the natural ecosystem along with the ability to be manipulated under controlled conditions in the laboratory, microcosms can overcome some of the short-comings of the previous two approaches; though remains liable to scrutiny of its own regardless. Depending on the chosen biological system cultivated, the same piece of equipment can serve as a microcosm or an experimental model. In these terms, it is good to note that models exist along a continuous spectrum spanning from *in situ* experiments to extremely simplified components of such systems. A number of approaches are reviewed below; highlighting those showing particular promise in the field of dental research. Interestingly, literature is scarce when it comes to *in vitro* models that study biofilm and enamel simultaneously.

1.6.2.1 *In situ* oral biofilm & caries models

Examining biofilm in its natural location is the most direct way of investigating it. Animal models have been ascribed in the past as the only method capable of assessing the entire caries process (White, 1992) but it has been shown that they do not always predict clinical results (Stookey *et al.*, 1985) and ethical issues regarding animal testing are evidence of the limitations of this approach. *In situ* models have been described as similar in many areas of simulating processes affecting caries (White, 1992). When investigating biofilms of the oral environments current models in use can be categorised into two main groups; those where the biofilm develops directly on to the subject's tooth, or those that rely on the development of biofilms on surfaces held within the mouth using either palatal devices or intra-oral splints. Substrata held within these devices are typically sterilised human enamel or dentine slabs, bovine enamel or hydroxyapatite discs. Using this methodology a number of *in situ* models have been developed to assess enamel caries (Zero, 1995; Gameiro *et al.*, 2009), dentinal caries (Lima *et al.*, 2008), fluoride penetration through dental plaque (Watson *et al.*, 2005) and to monitor the effect of preservatives on oral biofilms (Arweiler *et al.*, 2014). However, natural systems are often difficult to deal with directly, and it may not be possible to sample or to employ instruments in a way that can give reproducible and reliable results. The natural variation in biofilms militates against reproducibility. In this way *in situ* models are generally not used in primary screening processes due to the small number of replicates permitted but do constitute an important evaluation tool for undertaking research in the complex milieu of the mouth. A key disadvantage is that *in situ* models are not suitable for testing experimental products due to concerns for patient safety.

1.6.2.2 *In vitro* biofilm model systems

Existing biological models of dental caries vary markedly in their microbiological complexity. Laboratory models can range from pure cultures of cariogenic species, those that incorporate defined consortia of microorganisms or culture a microcosm. Defined consortia are often used to investigate ecological phenomena that would otherwise be difficult to measure for reasons of complexity and culturability and make use of simplified combinations of organisms that are amenable to growth on artificial media whilst also being readily identifiable once cultured (McBain, 2009). Pure culture, like other reductionist approaches, can have value in determining mechanisms of action or cause-and-effect relationships but lacks being representative of nature. A crucial advantage of taking environmental biofilm samples and bringing them into the laboratory setting is that environmental conditions can be controlled more precisely in a microcosm model. Control means the ability to vary conditions to determine the response of the system to environmental perturbation and since this type of investigation often affords some ease in comparison to those in the natural setting; results of these experiments are likely to be more reproducible. Approaches include homogenous models (Bradshaw *et al.*, 1989); chemostats with solid surfaces (Keevil *et al.*, 1987; Anwar *et al.*, 1989; Marsh, 1995); continuous irrigation systems (Dibdin *et al.*, 1976; Hudson *et al.*, 1986; Sutton *et al.*, 1994; Watson *et al.*, 1995), cell attachment models (Herles *et al.*, 1994; Li and Bowden, 1994), constant growth rate models (Gilbert *et al.*, 1989), artificial mouths (Wong and Sissions, 2001); simple batch culture systems (Guggenheim *et al.*, 2004) and more sophisticated steady state models such as the constant depth film fermenter (Atkinson and Fowler, 1974; Coombe *et al.*, 1981, 1982; Coombe, 1984; Peters and Wimpenny, 1988). All models are essentially compromises between the reality of the natural ecosystem and the simplification and controllability necessary to gain meaningful results in the laboratory setting. A suitable system can be chosen to meet the requirements of most types of investigations.

1.6.2.2.1 The constant depth film fermenter as caries – biofilm model

An ideal biological caries model holds dental hard tissue substratum; would allow long-term experiments, intermitted de- and remineralisation cycles and different treatments within one experiment along with providing sufficient biomass of biologically representative bacteria and sufficient reproducibility within and between experiments. It would also aim to mimic the nutrient flow characteristics of saliva by possessing a low medium volume: substratum surface ratio. A good biofilm model must enable aseptic sampling procedures and be sterilised easily. It should allow the study of microcosm, defined consortia or of pure culture inoculates. Samples must be discrete, representative, and as reproducible as possible; therefore the system should be able to generate large quantities of samples so that the statistical reproducibility of experiments can be examined. Additionally, samples should be removable with biofilm intact on its corresponding substrata so that it is disturbed as little as possible meaning the spatial organisation of the film is maintained. The constant depth film fermenter (CDFF) attempts to satisfy these criteria. The design of the units used throughout the studies of this thesis is described in detail in Chapter 2 (Section 2.1). A brief perspective of its theory and a review of its application will be discussed here.

The CDFF is a sophisticated model system capable of generating large amounts of individual biofilms. As a steady state system, it is a particularly powerful tool for studying microbiological phenomenon since it can be easily perturbed by factors of interest to obtain unequivocal observations of their effects. This system has been used to investigate factors that may influence the growth of bacterial communities in the oral ecosystem. It has been used to investigate biofilm structure and bacterial vitality in supragingival (Hope and Wilson, 2003) and subgingival (Hope and Wilson, 2006) microcosms; to study the efficacy of antimicrobials on biofilm (Kinniment *et al.*, 1996a; Pratten *et al.*, 1998a; Pratten *et al.*, 1999, 2000) and to study the

remineralisation (Deng *et al.*, 2005) and demineralisation of dentine by single species model biofilm (Deng and ten Cate, 2004). More recently a multispecies CDFF caries model inoculated with pooled saliva from 10 healthy individuals was described (Zaura *et al.*, 2011). Here the effects of fractions of edible shiitake mushrooms on biofilm cariogenicity and the demineralisation of bovine dentine compared against those of chlorhexidine and sterile water were examined. So far there is no evidence that the CDFF has been used to develop a model that holistically studies enamel demineralisation alongside aspects of a biologically relevant biofilm or to test the efficacy of fluoride in caries control using such an approach.

First described as the thin film fermenter (TFF) by Coombe *et al.* (1982) as an *in vitro* model of dental plaque and following further development (Peters and Wimpenny, 1988), the constant depth film fermenter (CDFF) is one of the most suitable *in vitro* methods of growing reproducible biofilms. During operation, cells attach to the substratum surface and proliferate to fill a predetermined recessed space. Excess material is removed from the upper surface of the film using a scraper blade. After a period the film becomes "steady state" as judged primarily by the microbial composition. Although it was not principally designed to study early biofilm formation or for studies that require a high shear-rate, some of the advantages of this model for experiments on oral biofilm include (Pratten, 2007):

1. It represents a good simulation of the oral environment. Improving on many *in vitro* models which involve immersing substrata in an excess of fluid, the fluid phase in the CDFF is continuously smeared in a thin layer over the biofilms / substrata.
2. Mechanical shearing of the biofilms by the scraper blades simulates the shearing present *in vivo* due to tongue movements, biting and chewing.

3. Exposures to test agents and additional nutrients such as sucrose can be highly controlled.
4. Sampling of the biofilm/substrata is easy and yields a large number of replicates.
5. Biofilms of different thickness can be investigated.
6. Pure or mixed cultures of bacteria, saliva or homogenised-plaque samples can be used as inoculum.
7. A number of substrata can be investigated.
8. The effects of the long-term exposure of biofilms to antimicrobial/anti-caries agents can be determined.

1.6.3 Modelling the Anticaries Efficacy of Fluoride

The effects of fluoride have been estimated by a wide range of *in vitro*, *in situ* and *in vivo* studies, helping to develop effective product formulations and understand its mechanism of action. These studies have focused on the availability of fluoride in toothpaste formulations, its bioavailability in saliva and remnants of disturbed biofilm, its reaction with the dental substrate to form loosely bound reservoirs as well as the ultimate reduction of mineral loss and increase in mineral and fluoride content of caries lesions (Tenuta and Cury, 2013). When adequately used, they can predict the anti-caries efficacy of formulations well, giving support for clinical trials to be pursued. They provide the basis for tests of equivalence between new products and those that are clinically proven and to investigate mechanisms of action (Zero, 2006).

Short-term *in vivo* studies provide important information of the expected clinical effect of toothpastes by assessing the bioavailability of fluoride during their use in the natural setting. *In situ* studies typically utilise highly cariogenic *in situ* conditions or extensively demineralised enamel samples. Conditions are chosen with an aim to

reduce the time required to estimate the anti-caries effects or highly sensitive tools for the detection of enamel de- and remineralisation are generally employed. These studies are devised to overcome one of the limitations of laboratory models; in other words they take into account the pharmacokinetics of formulations within the milieu of the mouth. *In vitro* studies attempt to simulate the intraoral effect of fluoride formulations, assessing the clinical efficacy of toothpastes or active components of it to predict distinct outcomes. This thesis will focus on the evidence derived from current *in vitro* models and how they have impacted our understanding of fluoride efficacy.

The first major contribution of laboratory methods in dentifrice research was the revelation that dentifrice fluoride needed to be available in solution to exert its anti-caries effects (Gron and Brudevold, 1967; Pader *et al.*, 1977; Stookey *et al.*, 1985). In the literature there is extensive evidence that pH cycling models are some of the most suitable for studying the anti-caries effect of agents, specifically fluoride toothpastes. Importantly, they can be considered relatively close to modelling the caries process as a gamut of de- and remineralisation cycles and have effectively presented dose responses to fluoride (Argenta *et al.*, 2003; Vieira *et al.*, 2005; Queiroz *et al.*, 2008; Stookey *et al.*, 2011). They are also better suited by addressing the finding that fluoride seemed to be largely active within its reactivity in early carious "white spot" lesions than in sound enamel (Koulourides and Cameron, 1980; Mobley, 1981). Further to this it has been demonstrated that the correspondence between pH cycling models and clinical studies is relatively good (Featherstone *et al.*, 1986; O'Reilly and Featherstone, 1987). The strengths and limitations of this kind of cycling approach in studying dental caries has already been reviewed in this thesis (see Section 1.6.1.2), here a number of particularly interesting pH cycling studies that incorporated fluoride efficacy testing will be briefly discussed.

Fluoride containing toothpastes were first tested in a pH cycling model developed by Featherstone (1986). Validated against an *in vivo* caries study, a sufficient dose response to fluoride was demonstrated against the backdrop of de- and remineralisation cycles relevant in nature. Further dose-responsive models to fluoride were developed using bovine enamel (Vieira *et al.*, 2005) and dentine (Esteves-Oliveira *et al.*, 2011). The latter study demonstrated dose-response to toothpastes high in fluoride concentration within a model predisposed to demineralising outcomes. Models of this type aim to assess fluoride's effect on the reduction of dental demineralisation while on the other hand; remineralising models allow the assessment of fluoride's enhancement of remineralisation (White, 1987). Dose-responses to low fluoride toothpastes have also been demonstrated, in this case within both demineralising and remineraling models (Queiroz *et al.*, 2008). The inhibition of enamel demineralisation when pretreated with saliva has been investigated in an automated pH cycling model (Page, 1991); the effects of fluoride toothpaste and fluoride-releasing materials on inhibiting dentine mineral loss in bovine dentine (Hara *et al.*, 2002) and combinations of fluoride toothpastes, APF gel and fluoride varnish have been tested in a remineralising model (Maia *et al.*, 2003; Paes Leme *et al.*, 2003). A pH cycling study also provided evidence of differential responses to fluoride in shallow and deep lesions (ten Cate *et al.*, 2006) and to test a new dentifrice containing triclosan, zinc citrate and fluoride (ten Cate, 1993).

It is important to note that non-biological *in vitro* model systems do not fully represent the mix of organic acids, the physicochemical environment of the biofilm / tooth interface such as bacterial cells and pellicle; nor do they account for the challenge faced by therapeutic agents in penetrating biofilm to reach enamel and aid in its remineralisation. The CDFF is particularly suitable for growing large quantities of reproducible biofilm, mimics the conditions of the oral environment well

and allows the simultaneous study of substrata and biofilm aspects. It has proven application in the field of dental research and has been used to study the antibacterial efficacy of a number of antimicrobials such as chlorhexidine (Kinniment *et al.*, 1996a; Wilson *et al.*, 1998; Pratten and Wilson, 1999; Deng *et al.*, 2004; Leung *et al.*, 2005; Zaura *et al.*, 2011); silver (Mulligan *et al.*, 2003) and gallium doped phosphate based glasses (Valappil and Higham, 2014; Valappil *et al.*, 2014); and fluoridated milk (Pratten *et al.*, 1999, 2000) as well as fluoridated mouthwashes containing triclosan, chlorhexidine digluconate and cetylpyridinium chloride (Pratten *et al.*, 1998c). Allan *et al.* (2002) were able to use a CDFF to demonstrate the antibacterial effect of particulate bioglass on *S. sanguis* biofilm impacting periodontal wound healing research. More recently the effects of cinnamon and clove oils on biofilm adhesion of zirconia and titanium coated dental implant materials were investigated in a CDFF biofilm model (Al-Radha *et al.*, 2013). In other healthcare fields the CDFF has been used to test; the antimicrobial effects of varying concentrations of sodium hypochlorite on *Listeria monocytogenes* within a multispecies biofilm (Norwood and Gilmour, 2000); the antimicrobial susceptibilities of chronic wound biofilms (Hill *et al.*, 2010; Malic *et al.*, 2011) and to study sessile human oropharyngeal microbiotas (Humphreys and McBain, 2013). Despite this, its application in mimicking the effect of topical fluoride regimens has been relatively limited so far.

Two studies up to now have incorporated solutions of fluoride pulsed in different ways, with different cariogenic challenges and complexities of biofilm. Hoogenkamp *et al.* (2003) used a modified CDFF to investigate the effects of both 0.5 mM (19 ppm) and 1.0 mM (9.5 ppm) sodium fluoride, continuously flowed along with mucin containing medium, on a nine-species defined consortium biofilm of either 300 μm or 600 μm thickness. This study showed that 28 mM glucose pulsed once daily enriched the growth of *S. mutans* and *L. casei* and fluoride had no detectable inhibitory effect

on this outgrowth at either concentration within the nine-species consortium. Reduced acidogenicity was demonstrated in 600 μm thick biofilm exposed to fluoride; pH response curves showed baseline pH was higher and the pH drop was less severe. Similarly, fluoride had no effect on the viability of single species *S. mutans* biofilm grown in a CDFF model but did effect acid production (Deng *et al.*, 2005). Here biofilm was exposed to a cariogenic challenge of 2% sucrose (58.4 mM) supplied four times daily for 30 minutes. 135 ppm sodium fluoride applied twice daily reduced lactic acid production by 75% after three days while when in combination with 0.2% chlorhexidine viability was reduced by 80% and lactic acid content by 93% after one day. The viability and lactic acid content decreased below detection limits (0.01% of original viability values and 0.21 nmol acid/μg protein respectively) in subsequent days and remained this way until the end of the experiment in the mixture group. The reduced lactic acid production and viability remained stable until the end of the experiment in the fluoride only group. Transverse microradiography (TMR) of the dentine samples post experiments revealed that partial remineralisation occurred in the fluoride group but lesions were deeper, while nearly complete remineralisation was seen in the mixture group (Deng *et al.*, 2005). Interestingly, secondary lesion bodies (lamination zones) were observed in fluoride exposed dentinal grooves after twelve days. Demineralisation of these secondary lesions, and levels of remineralisation within original lesions, was more pronounced by the twentieth day. This study also fully described the modified CDFF used by Hoogenkamp (2003) developed in an attempt to improve reproducibility within studies comparing the effects of caries-preventative agents; further explained in Chapter 4.

The CDFF has also been used to study the effect of glass-ionomer cements (GIC) with and without fluoride on the growth of 300 μm thick *S. sanguis* biofilm (Hengtrakool *et al.*, 2006). A fluoro-aluminosilicate glass GIC containing 16% CaF_2 (by weight) released a substantial amount of fluoride and exerted a significant bactericidal effect

during the first five days of experimentation, yet growth was higher in this group in subsequent days until the end of the experiment. This early bactericidal effect was attributed to the release of both fluoride and aluminium ions since aluminium reportedly possesses antibacterial characteristics (Kleber and Putt, 1995). This is of particular significance since the GIC's ability to reduce the early growth of *S. sanguis* means initial plaque formation would be interfered. *S. sanguis* biofilms elicited a decrease in surface hardness of both cements comparable to that seen after immersion in lactic acid at pH 5 indicating some cement degradation. Indentation studies showed shallower indentations in fluoride containing GIC used as substrata within the biofilm model than in fluoride free GIC. In another study microcosm biofilm grown (from the saliva of one healthy donor) in a CDFF model investigated fluoride release from GIC and showed that high fluoride releasing GIC (Vitremer, 3M, ESPE) inhibits dentine demineralisation adjacent to restorations irrespective of gap width (Cenci *et al.*, 2009). Gaps at the margins of restorations can allow bacterial invasion and biofilm accumulation leading to secondary caries at the tooth cavity wall. Here 10% (292 mM) sucrose was pulsed for five minutes eight times daily with a 10 hour period of overnight rest and demineralisation of restored dentine was quantified using transverse microradiography. Marginal gaps of 250 μm and under were investigated and it was shown that the presence of fluoride released from this GIC within these gaps was enough to overcome potential demineralisation and the effect of gaps of these sizes. Using this biofilm model they were able to show that in the absence of fluoride released from this GIC, or from other sources, gap width affects the onset of secondary caries.

1.6.4 Techniques in Assessing Enamel Demineralisation

The need for the adequate identification and quantification of dental caries is paramount in making appropriate treatment decisions and in developing new and effective treatment strategies. Visual-tactile and radiographic examinations as modes

of diagnosis appear satisfactory in cases with substantial, cavitated caries lesions but do not have the sufficient sensitivity and specificity needed to effectively diagnose early non-cavitated caries, secondary caries and root caries. It has been shown that radiographs underestimate the size of lesions (van Amerongen *et al.*, 1992) and cannot fully elucidate enamel and superficial dentine caries when evaluating occlusal caries (McKnight-Hanes *et al.*, 1990). This is principally due to the large amounts of adjacent sound enamel. Along with the unavoidable hazards of ionizing radiation and the high false-positive detection of occlusal caries (Ricketts *et al.*, 1995) radiographs possess some key limitations. Since it is accepted that the caries process is a dynamic one, methods that allow the continuous detection, quantification and monitoring of caries lesions are of utmost importance in meeting the requirements of the preventative approach for treating of dental caries, aspired to in modern-day dentistry.

1.6.4.1 Quantitative light-induced fluorescence (QLF)

Demineralised dental enamel autofluoresces less than sound enamel and this phenomenon has been exploited to develop a sensitive apparatus for the assessment of dental caries (Bjelkhagen *et al.*, 1982; Sundstrom *et al.*, 1985). Enamel autofluorescence was first demonstrated under UV light conditions (Stubel, 1911) over a century ago. Subsequent studies were compelled by the prospect of its use in unravelling the composition and structure of dental tissues (Armstrong, 1963; Spitzer and Bosch, 1976; Alfano and Yao, 1981; Hafstrom-Bjorkman *et al.*, 1992) and it was proposed as a means to detect dental caries as a consequence (Benedict, 1928; Bjelkhagen *et al.*, 1982). Fluorescence is a result of a change in the characteristics of light caused by a change in the wavelength of the incident rays following reflection from the surface of a material. By fluorescing naturally, dental enamel and dentine both autofluoresce. The physical characteristics of dental hard tissue fluorescence

have been described (Armstrong, 1963; Spitzer and Bosch, 1976; Alfano and Yao, 1981). The type of fluorescence is dependent on the wavelength of the incident light. Near ultraviolet light is emitted as blue fluorescence, whereas incident light in the blue and green spectrum emits yellow and orange fluorescence and the incident light in the red or near infrared spectrum emits red fluorescence. The intensity of the reflected fluorescent light is proportional to the amount of material that causes the fluorescence. The difference between the fluorescence of the sound tooth and that of a caries lesion can be safely made visible by the QLF method.

Quantitative light-induced fluorescence (QLF) is a non-destructive technique based on the principle that the demineralisation of enamel or dentine results in a loss of its autofluorescence. Normal white light from an arc lamp passes through a blue filter with peak intensity of 405 nm, illuminating the chosen sample of enamel. Dedicated software is used to capture images with a yellow high pass filter of 520 nm and, in the case of the Inspektor-Pro, a charge coupled (CCD) camera or within the Biluminator system, a digital SLR camera. The loss of fluorescence within a lesion is compared with the adjacent sound tooth tissue and a quantifiable value (% loss of fluorescence and integrated value of loss in fluorescence over the lesion area per pixel) is calculated. Demineralised lesions are dark in appearance. The observed fluorescence loss is not caused by a reduction in fluorophores per se, but by a change in light scattering properties (de Josselin de Jong *et al.*, 2009), explained by the increased scattering coefficient of the lesion compared to that of sound enamel (de Josselin de Jong *et al.*, 1995). As the scattering coefficient increases, a decrease in mean free photon path implies that the chance of a photon being absorbed by a fluorophore and a fluorescent photon being emitted is lowered. The green fluorescence of teeth is thought to be caused by intrinsic fluorophores within the dentine structures particularly around the enamel-dentine junction (EDJ) (Angmar-Månsson and ten Bosch, 2001). Mantle dentine at the EDJ is responsible for containing the intrinsic

proteinacious fluorophores which in turn are responsible for the autofluorescence of teeth. Excitation light enters the highly mineralised enamel and strikes the mantle dentine at the EDJ causing fluorophores to fluoresce and emit a lower wavelength fluorescent light (Angmar-Månsson and ten Bosch, 2001; van der Veen *et al.*, 2002). Levels of green-emitting fluorophores are highest at the dentine enamel junction (EDJ) and distributed so that their localisation is greater within the dentine than in the enamel. Within the area of demineralisation, the light travels shorter distances into the tooth, and the view on the EDJ is blocked, therefore this type of fluorescence is an indirect measure of enamel porosity or lesion severity (Stookey, 2004). Results obtained using QLF are reproducible (Benson *et al.*, 2003b) and valid (Benson *et al.*, 2003a). Changes in fluorescence radiance and lesion area can be charted longitudinally to measure lesion development or repair and has been used to monitor de- and remineralisation of tooth tissue during/or after treatment either clinically (Angmar-Månsson *et al.*, 1996; Al-Khateeb *et al.*, 1997b; Ferreira Zandoná *et al.*, 2000) or in the laboratory (*in vitro*) (Al-Khateeb *et al.*, 1997a; Al-Khateeb *et al.*, 1997b; Al-Khateeb *et al.*, 2000).

1.6.4.2 Transverse microradiography (TMR)

Though transverse microradiography is a destructive technique and can only be used upon the completion of an experiment, it remains the gold-standard in dental research when measuring mineral changes in enamel *in vitro*. This technique is based on the measurement of X-ray absorption by a tooth section compared with the absorption by a simultaneously exposed standard (ten Bosch and Angmar-Månsson, 1991) and is widely used to determine mineral morphology of tooth samples in terms of volume percentage (vol %) present in the sample, as a function of depth of sample. First developed by Angmar *et al.* (1963), it has been widely used in the field of dental research. The TMR method was expanded to demonstrate that after de- or remineralisation the loss and gain of mineral could be quantified (Arends *et al.*,

1983a; Arends *et al.*, 1983b) and software was developed resulting in a TMR scanning system with a high speed and low appearance of errors (de Josselin de Jong *et al.*, 1987). The error made in detecting spatial detail and the error in the calculation of x-ray absorbance were elucidated (de Josselin de Jong and ten Bosch, 1985) so that the current optimised transverse microradiographic system could be developed.
Validation of QLF against TMR for quantifying demineralisation has been performed using a commercial QLF system (Inspektor Research Systems BV, Amsterdam, The Netherlands) (Al-Khateeb *et al.*, 1997a; Al-Khateeb *et al.*, 1997b; Hall *et al.*, 1997b; Ando *et al.*, 2001)) and is further discussed in Sections 3.4.2 and 5.4.3.

1.6.4.3 Non-contact surface profilometry (NCSP)

To date, there has been relatively little analysis of dental caries in tooth enamel using non-contact surface profilometry (NCSP) though the technique has proven useful for the non-destructive study of enamel erosion (Zhang *et al.*, 2000; Hemingway *et al.*, 2010; Theocharopoulos *et al.*, 2010). The protective effect of fluoride components on tooth enamel treated with hydrochloric acid (Hove *et al.*, 2006); the characterisation of surface demineralisation by mono-species bacterial biofilm activity (Cross *et al.*, 2009) and the efficacy of a novel anti-erosive polymer system (Gracia *et al.*, 2010) have been investigated using optical profilometry. Recently, this technique has been validated against TMR for the *in vitro* quantification of dental erosion by orange juice (Elton *et al.*, 2009) and sweetened, fruit-flavoured alcoholic beverages commonly known as 'alcopops' (Ablal *et al.*, 2009). Profilometric characterisation of surfaces has a long history within the field of engineering (Thomas, 1982b, a; Bernardin *et al.*, 1997) since the mechanical behaviour of materials is affected by the roughness of its surface. Contact surface profilometry has been used extensively in dental research (Ashmore *et al.*, 1972; West *et al.*, 1998; Heurich *et al.*, 2010; Fujii *et al.*, 2011) yet has been shown to leave clear visible scratches on surface softened dental enamel leading to a larger measured value of surface loss (Heurich *et al.*, 2010). This damage means this

technique would not be suitable for long term monitoring of the same area on a surface. Therefore, exploring the use of less destructive methods is worthwhile.

Non-contact surface profilometry quantifies surface loss and characterises the surface of a sample. An optical stylus travels across a sample measuring deviations in height along its path in the X and Y directions to capture a three dimensional topograph of its surface. A cone beam and chromatic aberration measure distances with great accuracy. As the name infers, the probe does not make any direct physical contact with the surface ensuring that no damage is made to the potentially softened or eroded material. Depending on sample size, data collection is fairly rapid and relatively large areas can be assessed at one time. A sum of Fourier sine and cosine components of different wavelengths are used to derive the line profile of a surface. Appropriate filter functions are used to separate roughness and waviness, describing the short and long wavelength components respectively.

Since, unlike erosion, caries develops as a subsurface phenomenon the application of this technique, and others like it, in the study of dental caries is limited to the initial stage of caries lesion formation wherein enamel porosity rises and to latter stages of outright cavitation. Profilometry distinguished a statistically significant increase in the depth of pits and pores of enamel in presumed sites of retention of exogenous acid or chelating agents *in vivo* (Whitehead *et al.*, 1997). Nonetheless, the unique ability of NCSP to measure nanometre scale surface properties non-destructively, and its ability to operate real-time under liquid conditions (Reed *et al.*, 2008), means it could be modified for use within dental practise for diagnostics (Cross *et al.*, 2009). No previous studies have examined the correlation between NCSP and TMR measurements when looking at enamel demineralisation mediated by biologically

relevant biofilm not to mention its correlation with QLF as an indicator of demineralisation.

1.6.4.4 Scanning electron microscopy (SEM)

By enabling researchers to relate surface morphology to the microanatomy of the tissue, imaging of dental enamel by scanning electron microscopy (SEM) has been extensive. Early work provided more detailed information about the specific structure of teeth than could be captured with light microscopy (Boyde and Lester, 1967; Hoffman *et al.*, 1969; Newman and Poole, 1974). Characterisation of dental surfaces with this technique has included studies of cleaned surfaces (Marshall *et al.*, 1975), dental calculus formation (Lustmann *et al.*, 1976), topical fluoride application (Duschner *et al.*, 1997), debonding of orthodontic brackets (Zarrinnia and Kehoe, 1995), vital bleaching (Josey *et al.*, 1996), lasing (Frentzen *et al.*, 1996), the effects acid-etch treatments (Silverstone *et al.*, 1975) and studies of erosion and abrasion (Grando *et al.*, 1996; Sorvari *et al.*, 1996).

SEM uses a focused beam of high-energy electrons to generate a variety of signals at the surface of solid specimens (Boyde and Jones, 1996; Kim *et al.*, 2013; Cohen *et al.*, 2014; Milly *et al.*, 2014). These signals reveal information about the sample including external morphology, chemical composition, and crystalline structure and orientation of the constituent materials of a sample (Goldstein, 2003). Accelerated electrons carry significant amounts of kinetic energy that dissipates when the incident electrons are decelerated in the sample by electron-sample interactions. Signals are produced including those that form the SEM image (secondary electrons), back scattered electrons, those used to determine crystal structures and orientations of minerals (diffracted electrons), those used for elemental analysis and continuum X-rays (photons/characteristic X-rays), visible light and heat. Secondary and

backscattered electrons are usually used for imaging the sample of interest; wherein secondary electrons show morphology and topography while backscattered electrons illustrate contrasts in composition in multiphase samples. X-rays are generated by inelastic collisions of the incident electrons with electrons in discrete orbitals of atoms in the sample. As the excited electrons return to lower energy states, they yield X-rays that are of a fixed wavelength related to a given element. Therefore, X-rays are characteristic of the elements in a mineral that is excited by the electron beam.

SEM analysis is considered to be relatively "non-destructive"; X-rays generated by electron interactions do not lead to volume loss of the sample, so it is possible to analyse the same materials repeatedly (Egerton, 2005). Yet unless the instrument is capable of operation in a low vacuum mode, an electrically conductive coating must be applied to electrically insulating samples for study in conventional SEMs. Low vacuum mode samples are often limited in magnification and resolution compared to the traditional SEM procedure. It is therefore, generally thought not possible to monitor the effects of treatments longitudinally on the same enamel surface. In a study of enamel surfaces under exposure to carbonated drinks, real-time changes in topography were resolved by scanning force microscopy on the nanometre scale by image subtraction to quantify tissue loss (Sollböhmer *et al.*, 1995).

1.6.5 Methods for Assessing Biofilm Cariogenicity

Biofilm cariogenicity has often been investigated by traditional microbiological culture to elucidate the bacterial constituents of dental biofilms; indirectly by determining the state of the underlying substrata; or by techniques to garner the acidity or acidic products within the biofilm matrix. The tradition of studying the microbiology of dental caries is a long standing one; nonetheless, recent discussion within the field has emphasised that understanding the functioning, rather than just the composition

of the microbial community, should be a high priority for caries microbiologists (Marsh, 1994, 2003a; Beighton, 2005; Nyvad *et al.*, 2013).

1.6.5.1 Microbial ecology

Regardless of the recent advances in molecular microbiology techniques and bourgeoning exploration of its uses, the rudimentary traditional culture of microorganisms has provided considerable information of their association with dental caries. Traditional studies of the microbial ecology of caries can be laborious, due mainly to the high species diversity of dental plaque. Its focus on cultivable microorganisms, once a strength is now a limitation since it is now known that many oral bacteria cannot be cultivated and cultured but are viable. Therefore, conclusions drawn are from an incomplete picture (Wade, 2002). As long-standing primary suspects of caries, plentiful research has focused on the study of species of mutans streptococci and lactobacilli. A trend further propelled at the height of belief in the "specific plaque hypothesis"; it is now clear that such a simplified microbiological approach could be misleading when trying to understand the aetiology of caries. For example, the relationship between caries and mutans streptococci is not outright. There is clear evidence that caries does not always develop even in the presence of relatively high levels of this organism and that there are also circumstances where caries occurs in its apparent absence (Marsh and Martin, 1999). This evidence must lead us to stress an important distinction; mutans streptococci may reflect a disturbance in the homeostasis of dental plaque that is usually associated with health rather than necessarily initiating the caries process. Once disturbed, the homeostatic balance can no longer maintain conditions of health and these changes would inevitably cause proportional changes in the constituents of the microbial community such as an outgrowth of a select cohort of bacteria and predispose a site to the caries disease process. Myriad acid-induced adaptation and selection processes mean the microbial community is prone to ecological disruptions (Takahashi and Nyvad, 2008,

2011). Prolonged and severe acidic conditions elicit the dominance of more acidogenic and aciduric bacteria including not only *S. mutans* and lactobacilli but also some strains of non-mutans streptococci, *Actinomyces*, bifidobacteria and yeasts (Takahashi and Nyvad, 2011). In this sense, culture of these groups of organisms is still worthwhile but their presence alone cannot be an automatic indication that they are caries causative.

When investigating the composition of dental plaque using traditional microbiological techniques, constituent organisms are grown under appropriate growth conditions, on a range of selective and non-selective agar plates, for various periods of time. Microbial colonies are then identified using common biochemical, serological and physiological tests. In the design of studies concerned with evaluating the microbiological aspects of the caries process, the chosen sampling approach is a key factor. Cross-sectional studies involve the sampling of sites with existing caries at a single time and are thereby limited to comment only on associations. After all, it cannot be fully established that the organisms present were a cause of the lesion degradation or were simply better able to colonise the lesion once it was formed. In contrast, longitudinal studies can reasonably establish cause-and-effect relationships but can be time-consuming and more expensive in comparison. Samples are regularly taken over a defined period in this type of approach and the most useful begin with clinically sound surfaces so comparisons can be made between the microflora within and between time-points, before and after lesions develop and between test agents/conditions. Both approaches have provided evidence of the role of plaque bacteria in dental caries wherein the microbial composition was related to the integrity of the underlying tooth surface (Marsh and Nyvad, 2008). Nonetheless, both designs are limited by the efficacy of current diagnostic technologies in the detection of sub clinical enamel demineralisation (see Section 1.6.4). Though the

methodological approaches employed can be problematic, cultivation studies are not out-dated. In order to fully elucidate what they are doing, we still need live bacteria.

1.6.5.2 Biofilm acidity & microbial acidogenicity

Measuring the plaque pH response to carbohydrate exposure is an important part of the overall assessment of its potential cariogenicity and this approach has been used frequently since plaque-pH measurements were developed by Stephan (1940). Literature on the various methodologies is abundant and extensive reviews by Edgar (1982), Edgar and Geddes (1986), Firestone (1982), Kleinberg *et al.* (1982); and Schachtele and Jensen (1982) discuss their relative strengths and drawbacks. Methods include; pH telemetry (Graf and Muhlemann, 1966; Imfeld, 1977), touch electrodes (Kleinberg *et al.*, 1982; Scheie *et al.*, 1992) and sampling/scraping (Frostell, 1970; Loesche *et al.*, 1981) based methods. The sampling method gives relatively small pH falls since it measures superficial and homogenised dental plaque collected mainly from the buccal and lingual parts of the teeth i.e. from easily accessible crown or root surfaces. Since it does not measure pH changes in plaque from caries-prone areas, the value of the method has been questioned (Imfeld, 1977). On the other hand, the micro-touch method measures interdental plaque pH at accessible areas. This method permits repeated and direct readings from individual sites since the electrode is placed in continuous contact with the plaque until a steady reading is obtained (Scheie *et al.*, 1992). Values gathered from telemetry are generally comparatively lower than those measured by other techniques since it involves electrodes coated in plaque that record pH changes in interdental regions underneath an undisrupted plaque. All three methods have been evaluated comparatively in the investigation of plaque pH responses to starchy foods (Lingstrom *et al.*, 1993). Here, as expected, a large degree of variation was found in plaque pH values measured by each method. Since they measure pH at different locations of the dentition and at

different depths in the plaque, the methods may yield differing results with respect to both pH fall and pH recovery. Some studies elucidated that the mean minimum pH values obtained with the telemetric method were generally 0.7 pH units lower than when using sampling methods (Birkhed *et al.*, 1978; Jensen and Schachtele, 1983). Lingstrom *et al.* (1993) showed a larger mean difference between the two methods of 1.5 pH units. Telemetric methods provide information about only one site of the dentition, but the site is in an area where caries frequently occurs.

It has generally been assumed that the concentration of individual organic acids influences plaque pH greatly and in 1956, it was demonstrated that a significant correlation exists between pH and the lactate concentration of plaque (Carter *et al.*, 1956). It was concluded in this study that the influence of other acids was most likely negligible. In contrast, Geddes (1975) later found that the pH drop in glucose exposed plaque was due to lactic acid production whilst the major acids produced at neutral pH were volatile acids; mainly acetic acid. This finding has been confirmed extensively in the literature (Edgar, 1982; Vratsanos and Mandel, 1982; Distler and Kroncke, 1983; Geddes *et al.*, 1984). Currently, perhaps the most common method for determining profiles of the acids in dental plaque samples is by capillary electrophoresis (White *et al.*, 1995; Damen *et al.*, 2002; Zhang *et al.*, 2004; Chen *et al.*, 2010).

1.7 IMAGING & QUANTIFYING BIOFILM FLUORESCENCE

The removal or disruption of dental plaque from the tooth surface is the current primary mode of preventing plaque-associated diseases such as dental caries and periodontal disease. Achieved chiefly by effective daily hygiene, any plaque that remains in harder to reach areas of the mouth poses the largest risk of leading to the onset of disease. Therefore, by highlighting areas that require more attention and identifying caries risk, the effective detection of such dental plaque is crucial in

disease prevention. The detection of endogenous fluorophores can yield information about the metabolic state and the existence of various microorganisms (Konig *et al.*, 1994) and in the case of dental caries, porphyrin detection may be a useful indicator of lesion activity, pathogenicity of plaque or the failure of a restoration. Studying this fluorescence could provide an objective way of ensuring all infected dentine has been removed from a carious cavity as opposed to the current widespread use of visual or tactile methods which assumes that darkened or softened dentine is normally carious. In addition visualising biofilms in this way could provide a useful application in the study of plaque coverage and in assessing therapies used to reduce plaque (Pretty *et al.*, 2005; Coulthwaite *et al.*, 2006).

Biomolecules containing intrinsic fluorophores, such as; porphyrins, the amino acids tryptophan and tyrosine, the coenzymes NADH, NAPH and flavins cause cells and tissues to autofluoresce. Each type of fluorophore possesses distinct absorption and fluorescence maxima (Table 1.1) dependent mainly on structure and composition. It has been reported that dental plaque can fluoresce red when illuminated with blue light at a wavelength of 405 nm (Coulthwaite *et al.*, 2006; Thomas *et al.*, 2008). Little is known about the precise origin of this autofluorescence but red fluorescence appears to be associated with porphyrins, specifically protoporphyrin IX (Konig, 1993), as a by-product of bacterial metabolism (Lennon *et al.*, 2002). It has been found coating enamel surfaces; in particular within dental plaque (Konig *et al.*, 1993; Pretty *et al.*, 2005; van der Veen *et al.*, 2006), calculus (Heinrich-Weltzien *et al.*, 2003) and in cases of active caries (Lennon *et al.*, 2002; Shigetani *et al.*, 2008). As derivatives of the tetrapyrrole molecule, porphin, porphyrins are the intermediate products in metalloporphyrin heme (ferroprotoporphyrin), hemin (ferriprotoporphyrin) and chlorophyll synthesis. The most important porphyrins in biological systems are protoporphyrin IX, coporphyrin III, uroporphyrin III, and hematoporphyrin IX (Konig *et al.*, 1994).

Table 1.1 Absorption and fluroscence maxima of endogenous fluorophores present in bacteria of the oral environment. Adapted from Konig (1994).

Fluorophore	Solvent	Absorption (nm)	Fluorescence (nm)	Colour exhibited	Ref.
Tryptophan	H_2O	220, 280, 288	320 – 350	UV	Creed (1984)a
Tyrosine	H_2O	220, 275	305	UV	Creed (1984)b
NADH/ NADPH	H_2O	260, 340	470	Blue/green	Lee (1974), Salmon (1982)
Flavins	H_2O	260, 370, 450	530	Green	Galland (1988)
Zn-coporphyrin	DMSO	411, 539, 575	580	Yellow/red	
Zn-protoporphyrin	DMSO	421, 548, 585	592	Yellow/red	
Uroporphyrin	DMSO	404, 501, 533, 568, 622	624	Yellow/red	
Coproporphyrin	DMSO	398, 497, 531, 565, 620	622	Yellow/red	
Protoporphyrin	DMSO	406, 505, 540, 575, 630	633	Yellow/red	

As reviewed earlier within this thesis, QLF™ technology has been validated for use in the research and assessment of caries both qualitatively and quantitatively and is generally well accepted. This technology can also be used to visualise the red fluorescence within biofilm by employing its excitation wavelength at 405 nm and capturing images in real-time for analysis via specially programmed software for the quantification of plaque. It has been found that the presence or absence of red fluorescence is a good indicator of general oral health and the specific intelligent design of the QLF™-D products aids in patient understanding (Waller *et al.*, 2003). Current research focuses on characterising which bacteria exhibit red fluorescence and on how bacterial interactions and growth conditions may affect it (Coulthwaite *et al.*, 2006; Lennon *et al.*, 2006; van der Veen *et al.*, 2006; Thomas *et al.*, 2008; Volgenant *et al.*, 2013). A number of microorganisms are capable of producing endogenous porphyrins such as protoporphyrin production by some *Prevotella and Porphromonas* species (Shah *et al.*, 1979; Shah and Collins, 1990); red fluorescent porphyrins by *Propinibacterium acnes* of the skin (Cornelius and Ludwig, 1967; Kjeldstad *et al.*, 1984; Johnsson *et al.*, 1987), *Clostridium*, *Bifidobacterium*, *Actinomyces* (Brazier, 1986), *Bacillus thuringiensis* (Harms *et al.*, 1986), and

Staphylococcus aureus (de la Fuente *et al.*, 1986). Yet, previous work shows that not all microorganisms cultured on agar plates fluoresce (Konig *et al.*, 1993; Konig *et al.*, 1994) and that *S. mutans* does not exhibit red, but it does exhibit green fluorescence (Konig *et al.*, 1993; Lennon *et al.*, 2006). However, a recent study demonstrated that all 11 bacterial strains associated with caries and periodontal disease grown on tryptic soy agar alone failed to autofluoresce when visualised with QLF™-D (Volgenant *et al.*, 2013). Yet, when the growth media was supplemented with spinach extract (chlorophyll) red autofluorescence was observed from 7 of the 11 species including *S. mutans* and *L. salivarius*. *S. mutans* also exhibited red fluorescence when supplemented with blood, hemin and vitamin K. Only *Parvimonas micra* fluoresced red when the growth medium was supplemented with sheep blood. This work provides evidence that the red autofluorescence observed in mature dental plaque is likely related to the intrinsic metabolic products of the oral biofilm on a whole, and not inherently to the presence of the specific bacteria. It sheds light on the substrate inducible red autofluorescent abilities of caries important microorganisms, such as *S. mutans*. How the interactions of specific bacteria may influence red autofluorescence has been little researched save for observations that *Parvimonas micra* fluoresces red only in the immediate presence of *Porphyromonas gingivalis* (van der Veen *et al.*, 2006).

It has been confirmed that there is a significant association between the red fluorescence and the cariogenic properties of microcosm oral biofilm visualised *in vitro* using QLF™D (Lee *et al.*, 2013) as well as that this technology shows promise in monitoring the maturation of microcosm oral biofilm (Bittar *et al.*, 2014; Kim *et al.*, 2014). In the former study, microcosm biofilms from one healthy donor, propagated on bovine enamel specimens using 24-well culture plates, were grown in the presence of 0.05%, 0.1%, 0.2% and 0.5% sucrose to obtain biofilm ranging in cariogenicity. QLF-biofilm images were captured after 10 days and total and aciduric bacterial

counts were elucidated, along with measurements of biofilm pH and enamel microhardness. Similarly, Kim *et al* (2014) propagated biofilm in the same way but monitored its red fluorescence over the course of 10 days exposed to 0.5% sucrose only. Red fluorescence increased over time and as the biofilm cariogenicity increased, as indicated by increased damage to the enamel and dose-dependent lowering of pH; which was below critical pH in the 0.5% sucrose biofilm tested. Biofilm red / green fluorescence (R/G) ratio values were significantly correlated only with aciduric bacteria ($r = 0.83$, $p < 0.0001$ and $r = 0.85$, $p = 0.001$, in Lee *et al*, 2013 and Kim *et al*, 2014 respectively) and not with the total bacteria of the biofilm on a whole suggesting that the red fluorescence is associated with how the bacteria are behaving and less with the quantities of bacteria (Lee *et al.*, 2013). However Kim *et al* (2014) reported that the correlation between total bacteria and R/G ratio was statistically significant ($r = 0.74$, $p = 0.001$). In this way, findings from this work support earlier suggestions (van der Veen *et al.*, 2006; Volgenant *et al.*, 2013) that the autofluorescence of the biofilm is possibly induced by the characteristics its bacterial constituents are employing. QLF-D appears to have potential as a device for plaque assessment, but evaluation should not simply focus on the notion of identifying quantities of specific causative microorganisms but concentrate on its use in indicating the metabolic activity of biofilms.

1.8 Summary and Aims of Study

Despite being largely preventable, and some evidence of decline following increases in the use of fluorides, dental caries remains a public health concern; particularly among underprivileged groups in both developed and developing countries. Caries is the localised demineralisation of tooth enamel when biofilm bacteria metabolise dietary carbohydrates into acids. It is, therefore, important to employ a holistic approach in its study whereby the cariogenicity of biofilm alongside the progression of demineralisation in the associated enamel is investigated yet, in reality; few models

incorporate such an approach. Non-biological *in vitro* model systems do not fully represent the mix of acids, nor do they account for the challenge faced by therapeutic agents in penetrating biofilm to reach enamel and aid in remineralisation. The constant depth film fermenter (CDFF) produces fixed thickness biofilm and can mimic the oral environment with proven use in the field of dental research yet its application to test the efficacy of fluoride in a biologically relevant, multispecies, context has been limited. Mounting evidence supports the suitability of quantitative light-induced fluorescence (QLF) as a useful tool for assessing enamel demineralisation but so far has not been evaluated *in vitro* in a microcosm biofilm model. Furthermore, its use in investigating the biofilm fluorescence of cariogenic dental plaque shows promise. The application of non-contact surface profilometry (NCSP) in the analysis of dental caries has been relatively limited, primarily since caries develops as a subsurface phenomenon though the surface associated changes during the initial stage of caries lesion formation could be characterised using this method. Additionally, in this programme of research it could prove useful to corroborate potential erosion elucidated by TMR in the evaluation of the biofilm model developed. No previous studies have examined the correlation between TMR measurements and NCSP when looking at enamel demineralisation mediated by model biologically relevant biofilm not to mention with QLF as an indicator of demineralisation.

Therefore, the specific aims of these studies were as follows:

1. Develop a biologically relevant biofilm model capable of generating dental caries lesions rapidly with reproducibility.
2. Test the use of the model to evaluate the effect of fluoride in caries control.
3. Investigate the *in vitro* use of QLF to assess enamel demineralisation.
4. Investigate the *in vitro* use of NCSP to characterise caries associated enamel surface changes.
5. Investigate biofilm fluorescence as an indicator of biofilm cariogenicity.

1.9 THESIS OUTLINE

Following the review of the literature relevant to the scope of this study, materials and methods common throughout the empirical work are summated in Chapter 2 whilst specific experimental design is described within the body of each subsequent empirical chapter (Chapters 3 – 7). The development of a biological caries model utilising CDFF technology and evidence of its ability to produce caries is demonstrated within Chapter 3 wherein the effect of sucrose concentration on caries progression is investigated. Chapter 4 presents the development of a novel model system improving upon existing approaches in modelling biofilm. Modelling the effects of low-level fluoride, continuous fluoride exposure and the effects of frequency of fluoride administration are described in Chapters 5 and 6. Chapter 7 explores the use of QLF in visually imaging dental plaque biofilm and its potential in investigating the microbial ecology of biofilm. Chapter 8 concludes the thesis, offers discourse and proposes ideas for future work.

Chapter 2 Common materials and methods

2.1 The Constant Depth Film Fermenter (CDFF)

A number of CDFF units were used throughout these studies (Figure 2.1); all with the same basic assembly (Figure 2.2). Each fermenter consists of a stainless steel turntable which holds a number of polytetrafluoroethylene (PTFE) sampling pans, the number depending on the size of the device. The pans are located symmetrically around the rim of the turntable and placed flush to its surface using a specifically designed flat-ended tool (Figure 2.4a). Each sampling pan has five cylindrical holes (Figure 2.3), 5 mm in diameter, with PTFE plugs that can be accurately recessed to a specific depth using a machined and calibrated stainless steel tool (Figure 2.4c). Biofilms form in the recessed space created meaning five replicate biofilms are obtained when sampling each sample pan. A threaded hole in the centre allows each

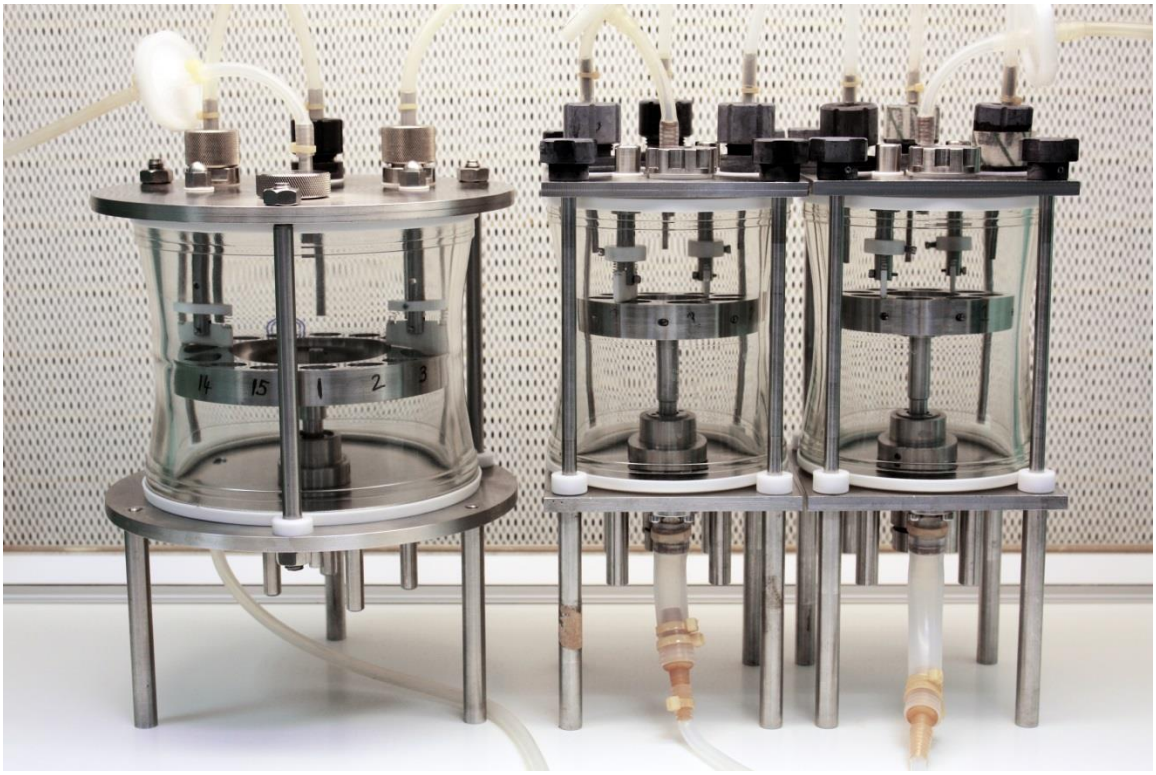
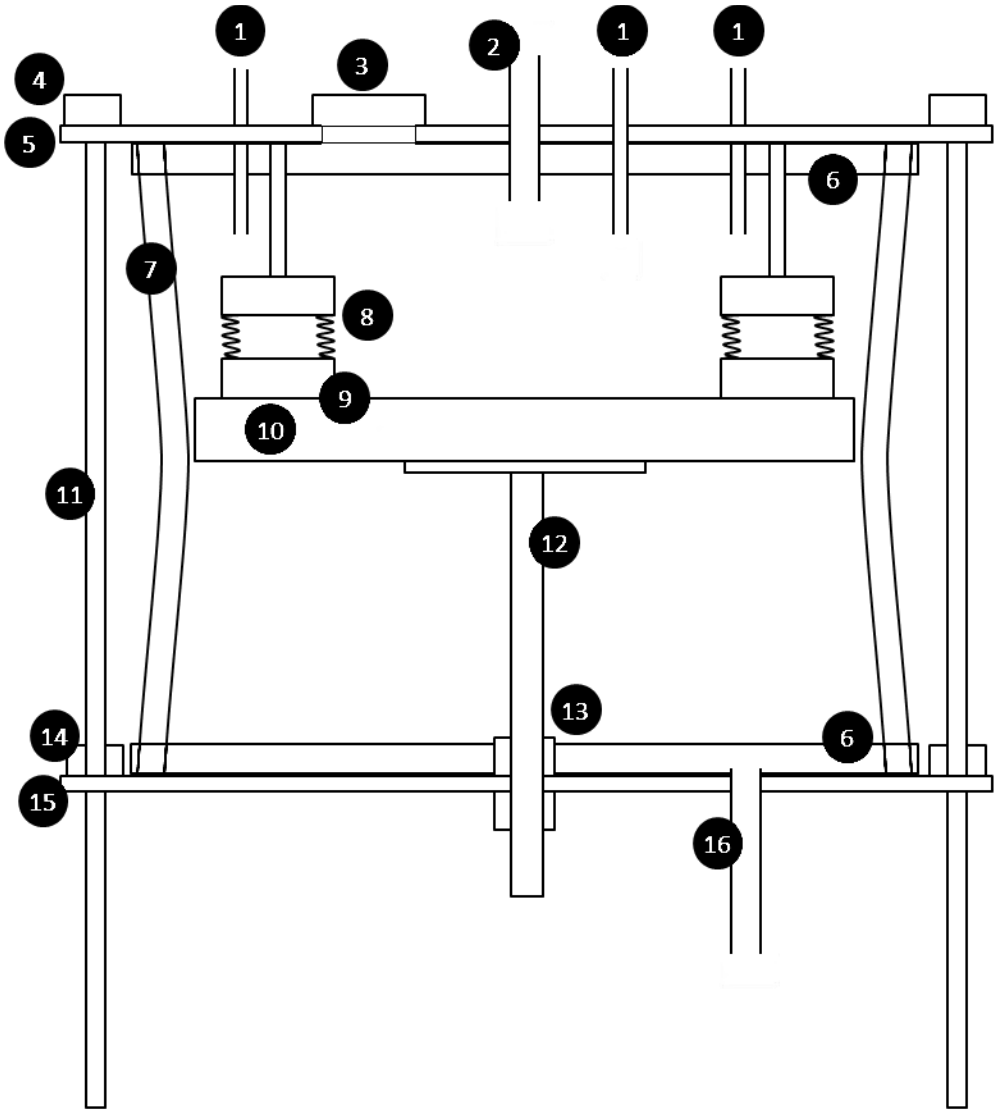


Figure 2.1. Constant depth film fermenters (CDFFs) fitted with appropriate tubing and air filters. Photograph shows 15-pan system (75 biofilm replicates) on the left and two 8-pan units (40 biofilm replicates each) on the right.

pan to be removed or replaced using a tool with a complimentary threaded end (Figure 2.4b). The turntable is housed within a glass vessel with stainless steel plates above and below, and held securely by threaded bolts fastened onto stainless steel tie rods (Figure 2.2). PTFE seals above and below the glass vessel along with PTFE spacers at the base of each tie rod aid in securing the assembly in place. The top plate has an entry port for sampling, inlets for introducing inoculated medium and sterile medium for producing biofilm as well as inlets for the entry of solutions expected to influence the caries process such as solutions of sucrose and fluoride slurries. Gases can be introduced directly into the vessel via the air inlet in the top plate. In the experiments of this thesis the CDFFs were exposed to atmospheric gases via a Hepa-Vent 0.2 μm pore filter (Whatman, Poole, Dorset, UK) to maintain sterility and sustain an aerobic atmosphere. The bottom plate has an effluent port for the egress of used medium. Silicon tubing (Fisherbrand, Fisher Scientific, Loughborough, UK) of different sizes was used to connect the CDFF units with the supply of media and other liquids and to the effluent reservoir. This type of tubing is non-toxic, durable, flexible, can be autoclaved and contamination can be seen easily within its bore. Specialised silicon pump tubing (Watson-Marlow Ltd., Falmouth, Cornwall, UK) was used within the peristaltic pumps, calibrated to deliver fluids at specific flow rates. The motor-driven turntable is connected to a central axle and bearing assembly which rotated at 3 revolutions per minute as controlled by a 0- to 15-V DC power supply unit. During the course of each experiment the turntable rotated beneath two spring-loaded PTFE scraper blades, smearing incoming fluids over the PTFE pans and maintaining the biofilm at a constant depth. The springs mean that the blades can fit closely against the surface of the rotating turntable and sample pans and efficiently remove biofilm once it grows above the surface of the pan.



1 Medium inlets; spatially distributed
2 Gas inlet
3 Sampling port with threaded lid
4 Threaded bolt
5 Top-plate
6 PTFE seal
7 Glass housing
8 Spring
9 PTFE scraper blade
10 Turntable
11 Tie rod
12 Central axle
13 Bearing housing
14 PTFE spacer
15 Bottom-plate
16 Medium outlet

Figure 2.2. Schematic of a vertical section through a typical glass-bodied CDFF.

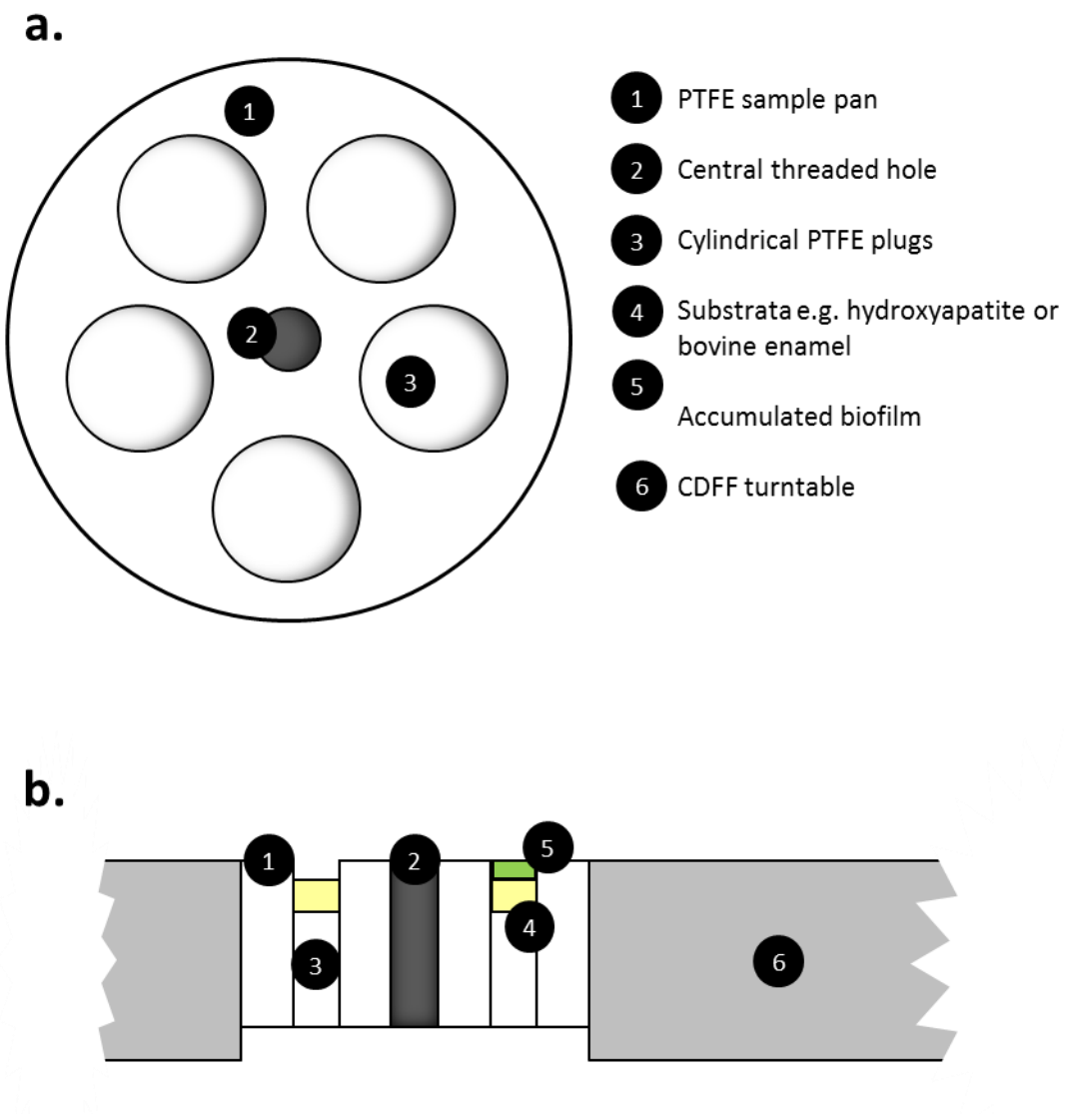


Figure 2.3. A closer look at a CDFF sample pan (a) in aerial view and (b) in cross-section within a CDFF turntable.

The 15 sample pan CDFF is 230 mm wide, has a turntable diameter of 150 mm and holds a total of seventy-five substrata discs. A matching pair of smaller CDFFs was commissioned (Mr Jeffrey Abbot, West Kirby, Merseyside). These could fit alongside one another within a standard laboratory incubator (IP250-U, LTE Scientific Limited, Oldham, UK). The width of these fermenters was 140 mm. Each turntable had a diameter of 100 mm holding eight PTFE sample pans and yielding a total of forty individual ~5 mm substrata discs to support the growth of aliquots of biofilm.

Figure 2.4. Tailor-made tools for; (a) placing PTFE pans flush to the turntable surface, (b) extracting and replacing PTFE pans within the CDFF system and (c) tool used to create recessed area for biofilm to accumulate within pan.

2.2 Saliva Pool

Whole human saliva was used as an inoculum in order to obtain multispecies biofilm representing the micro-organisms commonly found in the oral cavity. Unstimulated saliva samples were collected from local research staff volunteers (n = 23) comprising a mix of non-smokers and smokers, none of whom had been prescribed antibiotics 2 months prior to donation (Ethical Approval: University of Liverpool Research Governance Office, Physical Interventions Sub-committee, Ref: RETH000377). 3 mL aliquots were taken from each saliva sample, combined and homogenised by magnetic stirring. An equal volume of sterile skim milk powder (Oxoid, Basingstoke, UK) solution was added to the saliva pool at a final concentration of 10% w/v to act as a cryoprotectant (Cody *et al.*, 2008). The resulting suspension was dispensed into 1.8 mL aliquots and stored in cryogenic vials (Greiner Bio-One, Gloucestershire, UK) at -80ºC until required. Preparation of the saliva pool was undertaken speedily in order to minimise the cytotoxic effects of oxygen on the strict anaerobic microorganisms.

2.3 Enamel Specimen Preparation

Work was done to prepare discs from extracted bovine enamel teeth for use within the studies of this thesis. Bovine teeth were extracted at an abattoir and stored in 10% thymol solution; a method previously shown as acceptable to prevent bacterial growth

(Kinney et al, 1993). A number of considerations were taken into account when producing the enamel discs suitable for insertion into CDFF sample pans. They must be 4.9 mm ± 0.1 mm in diameter, be planar-parallel and have a relatively smooth surface so the volume of biofilm accumulated upon them was standardised between experiments. A water-cooled diamond-tipped core drill was used to cut cores of enamel from bovine teeth held in place, at first, by clamps alone. However, for the majority of the experiments, pre-made enamel disc (Intertek 4-Front, Chester, UK) were used in order to improve conformity and save time. Prior to use in experiments involving enamel, bovine enamel discs (Intertek 4-Front, Chester, UK) were checked for surface & fluorescence abnormalities using the QLF Inspektor™ Pro (Inspektor Research Systems BV, Amsterdam, The Netherlands). Enamel discs without such abnormalities were selected and half painted with acid resistant nail varnish (Nailfinity® shade 101 Crystal Clear, Max Factor, Proctor & Gamble, Weybridge, UK). A small cut was made into enamel discs along their depth on the side protected with nail varnish (Figure 2.5) using a high-speed handpiece (connected to a Marathon-N7 micromotor; Saeyang Microtech, Daegu, Korea) with a diamond coated cutting disc attachment (Skillbond Direct Ltd., High Wycombe, UK).

2.4 Sample Pan Preparation

In the experiments of this thesis either hydroxyapatite (Clarkson Chromatography Products, South Williamsport, PA, USA) or pre-prepared bovine enamel discs (Section 3.3) were placed on each of the plugs in the pans before being recessed to 200 µm. All pans were marked in order to identify the different substrata so the identity of each replicate biofilm and substrata could be monitored based on positioning in relation to this reference mark. In experiments involving enamel, pans were prepared so that they contained three enamel discs and two hydroxyapatite (HA) discs (Figure 2.6). Any sample pans loaded with enamel were placed upright in a stack within a 30 mL

polystyrene container (Sterilin, Cambridge, UK) and packed for security with soft tissue or extra pans prior to sterilisation by gamma irradiation.

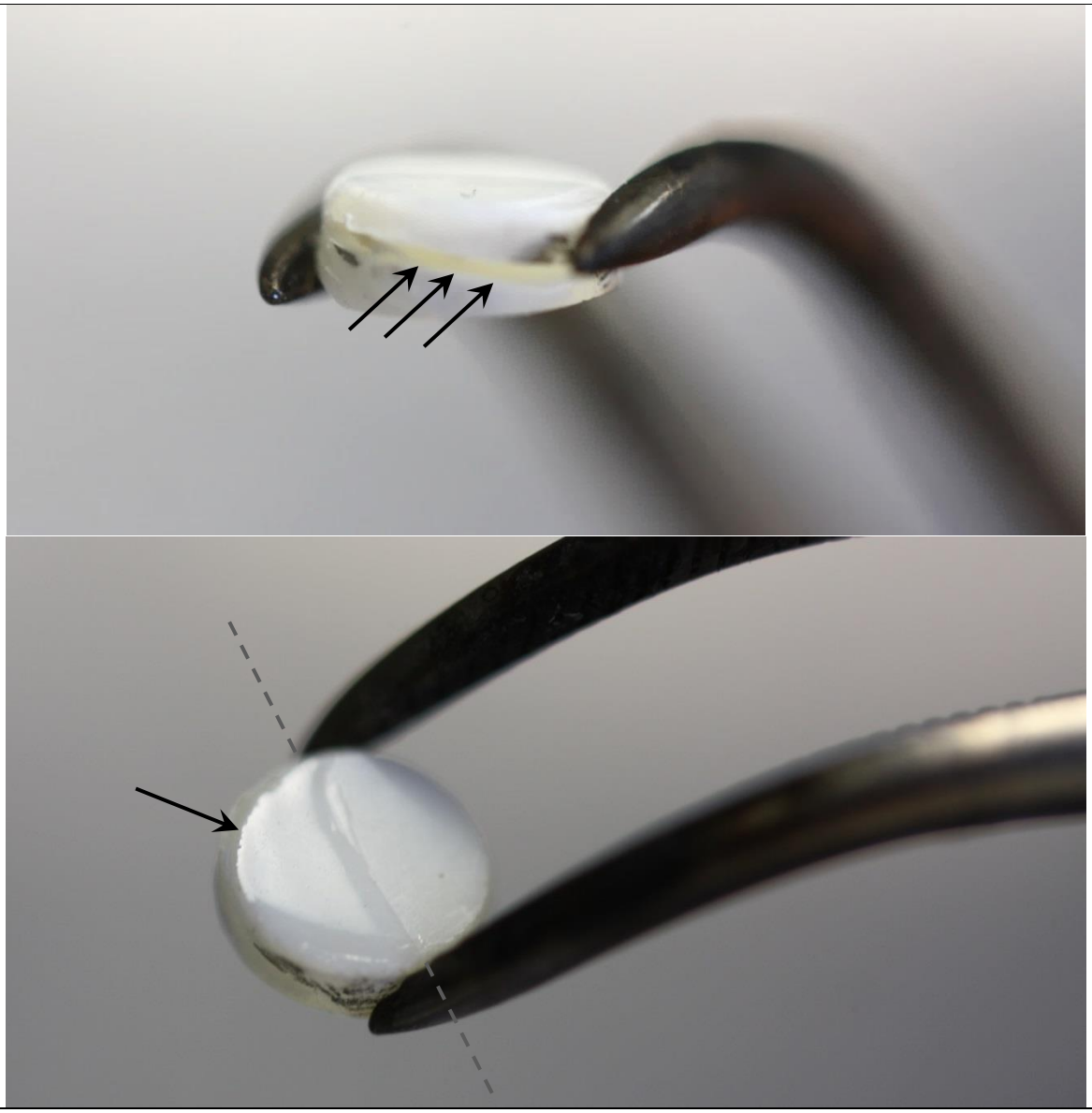


Figure 2.5. Bovine enamel disc with incision (pictured above, indicated by black arrows) and left half-painted with acid resistant nail varnish (pictured below, black arrow indicates nail varnish coated side).

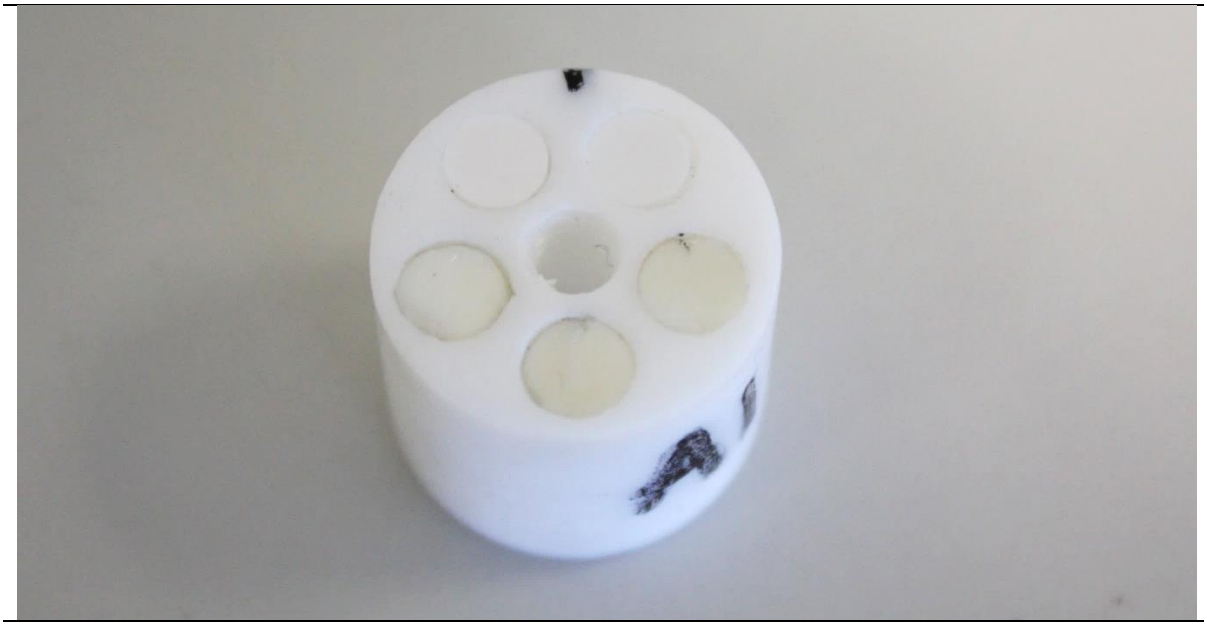


Figure 2.6. Typical sample pan containing two HA and three bovine enamel discs and marked for reference to distinguish sample identity.

2.5 Equipment Sterilisation Procedures

For initial experiments with HA samples, the CDFF was sterilised by autoclaving at 121ºC and 2200 mBar for 15 minutes (Touchclave Lab K200s, LTE Scientific Ltd., Oldham, UK). However, the pressure changes generated within the autoclave cycle often meant some hydroxyapatite discs were displaced during sterilisation; most probably due to boil-off occurring beneath the discs causing them to lift slightly. This meant the CDFF needed to be accessed post-sterilisation to replace individual discs into the correct location in the sample pan aseptically which nevertheless posed a contamination risk. Subsequent experiments used dry-heat sterilisation at 140ºC for 3 hours (Memmert UFB500, Memmert GmbH, Heilbronn, Germany). The sterility of this procedure was validated in the first instance. When preparing for experiments involving enamel as substrata the CDFF without sample pans was sterilised by dry-heat at 140ºC for 3 hours, using a fan assisted oven (Memmert UFB500, Memmert GmbH, Heilbronn, Germany). Whilst still hot from the oven the CDFF was placed in a sterilised laminar flow cabinet (Bassaire P5H Cabinet, Bassaire Ltd., Southamptom, UK) and the gamma irradiated sterile pans containing bovine enamel

samples were introduced aseptically. All other glassware and media components were sterilised by autoclaving at 121ºC for 15 minutes. Gamma irradiation to sterilise the enamel discs was done at a dose of 4,000Gy (4kGy) and a rate of ~3Gy/min for 18 hours (Gammacell 1000; Field Emission, Newbury, Berks, UK) at the Ronald Ross Building, University of Liverpool by Ms Caroline Broughton, Department of Clinical Infection, Microbiology & Immunology.

2.5.1 Determining the sterility of CDFF post oven sterilisation

To determine the sterility of the CDFF equipment after sterilisation by dry heat, two were sterilised in this way then located in a laminar flow cabinet. One litre of liquid Brain Heart Infusion (BHI) was poured aseptically into each CDFF via the sampling port in the top plate. After 5 minutes the liquid was drained from each into sterile universals and sterile glass durans. These aliquots were incubated in duplicate under a number of conditions to encourage the growth of all possible contaminants i.e., two universals containing 20 mL BHI were incubated either aerobically, anaerobically and anaerobically with continuous agitation; all at 37ºC. Two universals contained 10 mL BHI, these were incubated aerobically with continuous agitation to encourage the growth of any microaerophilic microorganisms such as *Staphylococcus aureus*. After four days the contents of each universal was inspected for the appearance of growth or clouding. All samples remained clear. As an extra measure 20 μL of each sample was plated onto both blood and nutrient agar in duplicate and incubated for 3 days at 37ºC under aerobic or anaerobic conditions. All plates were clear of microbial growth.

2.5.2 Determining the sterility of irradiated pans

Each time a batch of sample pans were irradiated an extra pan was purposefully reserved within the sterile universal in order to test the success of the sterilisation procedure. 20 mL sterile BHI was added to the universal and the protocol to test sterility was employed as described in Section 2.5.1.

2.6 PRODUCTION OF BIOFILM

The CDFFs were inoculated in the same way in all experiments. One litre of a mucin-containing artificial saliva growth medium (Pratten *et al.*, 1998c) comprising Lab-lemco 1 g L^{-1}, yeast extract 2 g L^{-1}, proteose peptone 5 g L^{-1} (all Oxoid, Basingstoke, UK), type III hog gastric mucin 2.5 g L^{-1}, potassium chloride 0.2 g L^{-1} (all Sigma-Aldrich, Poole, UK), sodium chloride 0.2 g L^{-1} (BDH Ltd.; VWR, Leicestershire, UK) and calcium chloride 0.3 g L^{-1} (Acros-organics; Fisher, Leicestershire, UK) was prepared in an inoculation flask and autoclaved at 121ºC for 15 minutes to sterilise. The temperature of the flask was maintained at 37ºC by locating within the same incubator as the CDFF. A magnetic stirrer ensured continuous mixing while an aliquot of the pre thawed saliva pool was added. The inoculum was introduced into the CDFF at a flow rate of 0.72 mL min^{-1} using a peristaltic pump (Watson-Marlow Ltd., Falmouth, Cornwall, UK). After approximately 10 hours the inoculum was exhausted and a reservoir of sterile artificial saliva growth medium was pumped in at a flow rate of 0.5 mL min^{-1}; equivalent to the average salivary flow rate in humans of 0.72 L day^{-1} (Lamb *et al.*, 1991; Guyton *et al.*, 1997).

2.7 BIOFILM ANALYSES

2.7.1 Microbiological enumeration & identification

On specific days, sample pans were removed and all the biofilm-laden substrata were carefully extracted using sterile forceps. Enamel discs painted with nail varnish were reserved for enamel specimen analysis. The two unpainted enamel discs or hydroxyapatite discs laden with biofilm were vortex mixed for 30 seconds in 900 µL of phosphate-buffered saline (PBS) to disrupt the biofilm and the resulting cell suspension was serially diluted up to 10^6 in additional PBS. 20 µL of each dilution was cultured in quadruplicate on Blood Base No. 2 Agar (BA) (Bioconnections, Leeds, UK) containing 5% defibrinated horse blood (TCS Biosciences, Buckingham, UK) to enumerate viable bacteria in total colony forming units (CFU) present per mm^2 of

enamel disc. Additional solid selective growth media were used to isolate and enumerate particular organisms of interest, again in quadruplicate counts; *Streptococcus* spp. on Mitis Salivarius agar (MSA) (BD, Oxford, UK) supplemented with 1 mL^{-1} tellurite solution; total mutans streptococci group on Tryptone Yeast Cysteine agar (Lab M, Bury, UK) supplemented with sucrose (Sigma) and bacitracin (Sigma); *Lactobacillus* spp. on Rogosa agar (RA) (Oxoid, Basingstoke, UK). All plates were incubated at 37°C for 72 hours under anaerobic conditions (80% N_2, 10% CO_2, 10% H_2; Whitley MG1000 Anaerobic Workstation; Don Whitley Scientific Ltd., West Yorkshire, UK). Volatile sulfur compound producers were enumerated on Columbia agar base (Oxoid Ltd, Basingstoke, UK) supplemented with 1 gL^{-1} ammonium iron (III) citrate (BDH Ltd.; VWR, Leicestershire, UK), 1 mgL^{-1} hemin (Sigma) and 1.2 gL^{-1} glutathione (Sigma) incubated under the same anaerobic conditions for 168 hours (1 week). The blood-containing plates were incubated for a further 96 hours after counting to allow black pigmented anaerobes to develop the dark pigment to enable them to be enumerated. Following the incubation period CFUs for each bacterial group were obtained, first by identifying plates containing 30 – 300 colonies. Accurate counts were obtained in quadruplicate using an electronic plate counter (Stuart SC6 Colony Counter; Bibby Scientific Ltd., Staffordshire, UK).

2.7.1.1 Matrix Assisted Laser Desorption/Ionisation: Time-of-Flight Mass Spectrometry (MALDI –TOF MS) technique

MALDI-TOF MS was done at the Medical Microbiology Laboratories of the Royal Liverpool & Broadgreen University Hospitals under the supervision of Ms Karen Billingsley. A thin smear of each bacterial isolate, from 24 hour pure cultures, was deposited onto a MALDI plate in duplicate (Bruker MSP 96 target polished steel plate; Bruker Daltonik, Bremen, Germany) and allowed to air dry. Samples were overlaid with 1 µL of matrix solution (saturated solution of α-cyano-4-

hydroxycinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid batch number 255344) within 10 minutes of initial inoculation. The matrix sample was crystallized by air drying at room temperature. The organic solvent in the matrix solution extracts proteins from the organism which are predominantly ribosomal proteins, present in high concentrations. Measurements were performed under vacuum using a Microflex mass spectrometer (Bruker Daltonik, Bremen, Germany) with FlexControl software (version 3.0). Spectra were recorded in the positive linear mode (laser frequency, 20 Hz; ion source 1 voltage, 20 kV; ion source 2 voltage, 18.4 kV; lens voltage, 9.1 kV; mass range, 2,000 to 20,000 Da). For each spectrum 240 shots in 40-shot steps from different positions of the target spot (automatic mode) were collected and analyzed. Spectra were internally calibrated by using *Escherichia coli* ribosomal proteins. The spectra were imported into the BioTyper software (version 2.0; Bruker, Germany) and were analyzed by standard pattern matching with default settings. The MALDI Biotyper identifies microorganisms by analysing the expression of their intrinsic proteins using mass spectrometry. This mass spectral pattern of protein expression is compared with reference patterns in a database. The BioTyper database is regularly updated by Bruker and contains spectra of approximately 2,881 species. Results of the fingerprint analysis were categorised according to the manufacturer recommendations with scores ranging from 0 to 3. For each isolate, the highest score of a match against a spectrum in the database was used for identification. Scores below 1.7 were considered not to have generated a reliable identification; a score of above 1.7 was deemed as satisfactory identification of genus, and a score of $\geq$ 2.0 was acceptable for species identification.

2.7.1.2 Gram stain procedure

Each single bacterial colony grown on solid media described in Section 2.7 was emulsified in sterile saline on grease-free laboratory slides. The resultant bacterial smear was air-dried then heat-fixed. Slides were exposed to Crystal Violet dye (content ≥ 90%; Sigma-Aldrich, Poole, UK) for 1 minute then rinsed with deionised water before exposure to Gram's iodine for 1 minute followed by a second deionised water rinse. Slides were subsequently treated with 95% ethyl alcohol for 5 seconds and rinsed again with deionised water. Finally, slides were exposed to Safranin O dye (content ≥ 85%; Sigma-Aldrich, Poole, UK) for 45 seconds then washed with deionised water and blotted dry with bibulous paper (Fisher Scientific, Loughborough, UK). Bacterial smears were inspected for colony morphology using a light microscope under oil-immersion and to distinguish between isolates as Gram-negative or Gram-positive.

2.7.2 pH

pH was measured at room temperature using a combination pH electrode (Orion, Boston, MA) attached to a fully calibrated pH meter (Jenway model 3305, Jenway, Essex, UK). Solutions of pH 4 and 7 were generally used in calibrating the pH meter immediately prior to measuring sample pH.

2.7.3 Fluoride

Fluoride ion activities were measured at room temperature using an ion-selective electrode (Model 96-9, Orion Research Inc., Fisher Scientific, Loughborough, UK). 0.1 M fluoride stock solution (Orion 940906, Orion Research Inc., Fisher Scientific, Loughborough, UK) was ten-fold serially diluted to make up standard solutions ranging in concentration from 10,000 to 0.1 μM fluoride of 5 mL in volume. These standards were used to obtain calibration curves at least once daily before ascertaining sample fluoride measurements. Prior to probing any solution, be it fluoride standard or sample in question, 1 mL liquid to be measured was dispensed

into a clean vessel and 100 µL total ionic strength adjustment buffer (TISAB III, Thermo Electron Corp., Waltham, MA, USA) added, solution was thoroughly vortex mixed and meter readings were recorded in mV (Orion model 720A, Orion Research Inc., Fisher Scientific, Loughborough, UK). A precise 100 ppm fluoride standard complexed with 10% v/v total ionic strength adjustment buffer (TISAB III, Orion Instruments, Boston, MA, USA) was prepared and fluoride within was measured over the course of each day to determine possible drift and further validate the relation of mV to ppm calculation. The electrode was washed in deionised water and dried between sample measurements to prevent cross-sample contamination and immersed in a 2 mL solution of 1 µM fluoride standard with added total ionic strength adjustment buffer (TISAB III, Orion Instruments, Boston, MA, USA) at 10% v/v during periods of delay in use.

2.7.3.1 Determining fluoride in artificial saliva growth media

Effluent samples were obtained from the outlet port of the CDFF and were first analysed immediately using the electrode and protocol above. Aliquots of effluent at each time point were centrifuged at 6500 g for 30 minutes and the buffered supernatant was analysed for fluoride (Duckworth et al, 1987).

2.7.3.2 Determining fluoride in dental biofilm

Plaque samples were weighed, dried at 60°C for two hours, and reweighed (Duckworth et al, 1987). 1 mL deionised water was added to each sample and left overnight (Duckworth *et al.*, 1994) and fluoride concentrations of the aqueous extracts were measured using the electrode as above. In their 1994 study, Duckworth *et al* showed that on average distilled water extracted just as much fluoride from plaque samples as perchloric acid used extensively in their earlier work.

2.7.4 Quantitative Light Induced Fluorescence (QLF): biofilm imaging

A number of versions of the QLF™ technology were used throughout the course of the studies of this thesis as they became available during the process of device development. Whenever possible, white light and fluorescence photographs of laboratory generated biofilm were captured and fixed camera settings were kept consistent throughout. Early work involved the Inspektor™ Pro, an intra-oral camera for making QLF-images of individual element surfaces (Figure 2.7). Primarily used for research, it was the foundation of validating the QLF technology for the *in vivo* measurement of de- and remineralisation. The QLF-D Biluminator system (Figure 2.8) comprises an illumination tube (Biluminator; Inspektor Pro Research Systems, Amsterdam, The Netherlands) mounted onto an SLR camera (Canon model 450D; Canon, Tokyo, Japan) equipped with a 60 mm *f*/2.8 macro lens (model EF-S; Canon, Tokyo, Japan). Within the illumination tube is a ring mounted with eight violet-blue light-emitting diodes (LEDs; 405 nm ± 20 nm) and four white LEDs (broad spectrum, 6500K) with customised gel-filters in front of the camera lens. The camera system was controlled by, and images were captured and stored using, the dedicated proprietary software (C2 version 14; Inspektor Pro Research Systems, Amsterdam, The Netherlands). Prior to imaging, the camera's custom white balance was calibrated against a white card photographed under standard imaging conditions. Photographs were taken in a darkroom with fixed camera settings for each experiment unless otherwise stated (QLF photographs: shutter speed 1/10 seconds; aperture value *f*/2.8; ISO 200).

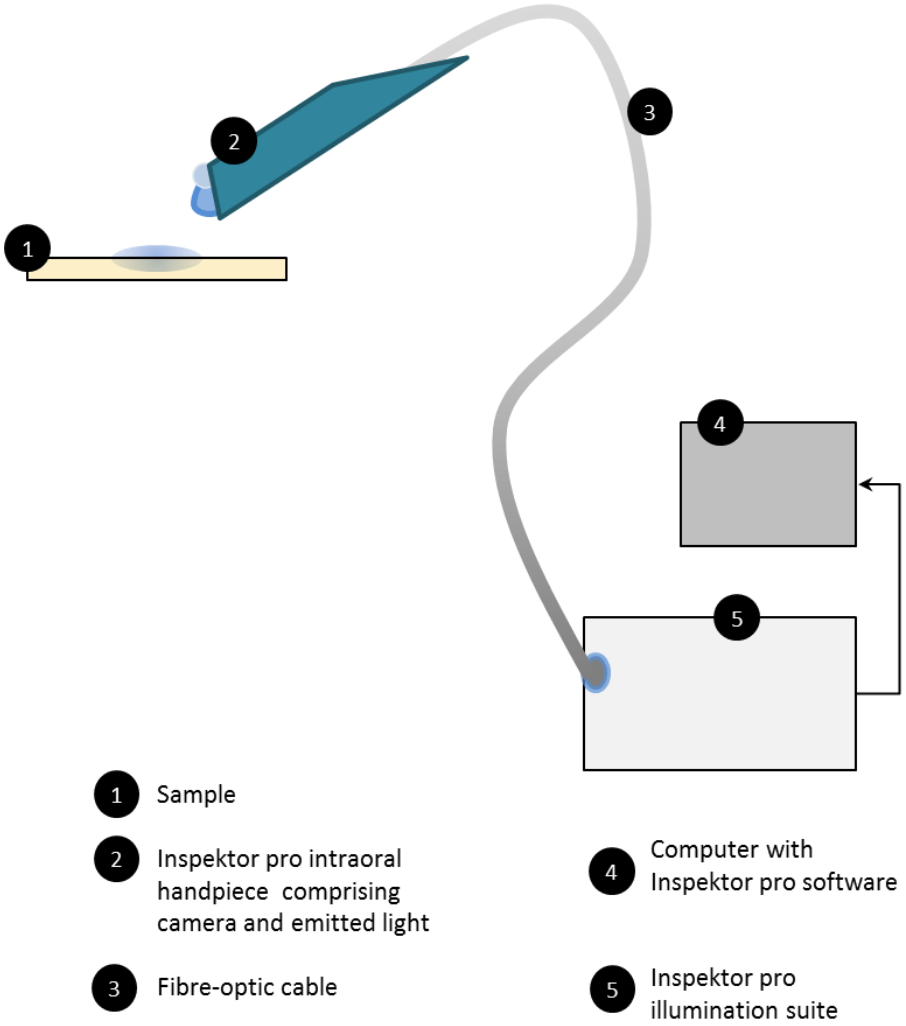


Figure 2.7. Simplified schematic of Inspektor™ Pro system comprising hand-held intraoral camera and 405 nm light transmitted via insulated fibre-optic cable.

Figure 2.8. Overview of QLF-D Biluminator system comprising Canon 450D SLR camera and fixed focal length macro lens (60 mm *f*/2.8) fitted with filter and finely polished illumination tube containing LED ring.

Prior to the routine use of the QLF-D Biluminator system a modified form that abided by the same principles was used to photograph dental plaque in the research setting under reproducible lighting conditions. Termed the QLF-*in vitro* system, it was a custom-made setup as described by Hope *et al.* (2011) (Figure 2.9). Briefly, it consisted of three LED lights (surface-mounted inidium gallium nitride light-emitting diodes; EWC 400 SC2C, radiant power 600 mW, 23° beam angle; E Wave Corp, London, UK) soldered onto the outside of copper rings. These were fixed inside an approximately hemispherical plastic bowl and angled so the emitted light converged on the sample placed centrally. The LEDs emitted violet light at wavelength band from 400 nm to a peak output at 405 nm. The copper rings acted as a heat sink, preventing overheating. A hole cut into the base of the bowl meant a camera (SLR; see description above) could be positioned above for capturing images whilst another hole cut into the side-wall allowed samples to be placed and manipulated easily. The LEDs were powered by a DC adaptor with an output of 5 V at 1.2 A. The light incident on the sample was measured as 220 W/m^2 irradiance by a photosynthetically active radiometer (PAR) with a cosine corrected detector (Q201 PAR with SD221Q Cos detector; Macam Photometrics Ltd; Livingston, West Lothian, UK). In order to minimise the transmission of light close to the excitation wavelength whilst maximising the transmission of the red part of the spectrum, a cut off filter (D007; Inspektor Research Systems BV) was placed in front of the camera lens.

2.7.4.1 Image analysis protocol

Both the Inspektor™ Pro system and C2 software use the same algorithms and results calculated by these methods are comparable. To quantify fluorescence colour compositions of the biofilm images captured, images were analysed using an open-source software package (ImageJ 1.43q; The National Institutes of Health, Bethesda, MD, USA, http://rsb.info.nih.gov/ij/). Images were opened with ImageJ and its red, green and blue (RGB) component colours were obtained by defining a region of

interest (ROI) using a circular selection tool around each biofilm containing sample plug (n = 5) of the CDFF sample pan and saved for repeat use throughout these studies. The colour attributes of the selected sample were obtained and exported into Microsoft Excel for data analysis. R/G ratios for individual biofilms were calculated by the division of red pixels with green pixels within the same ROI. Values were averaged and subsequently analysed for statistical significance. To determine the reliability of the image analysis method described above, two researchers independently analysed the image stacks from one experiment. This was to evaluate the possible variability associated with the manual selection of the ROI such as size and appropriate placement within the confines of the sample plug border.

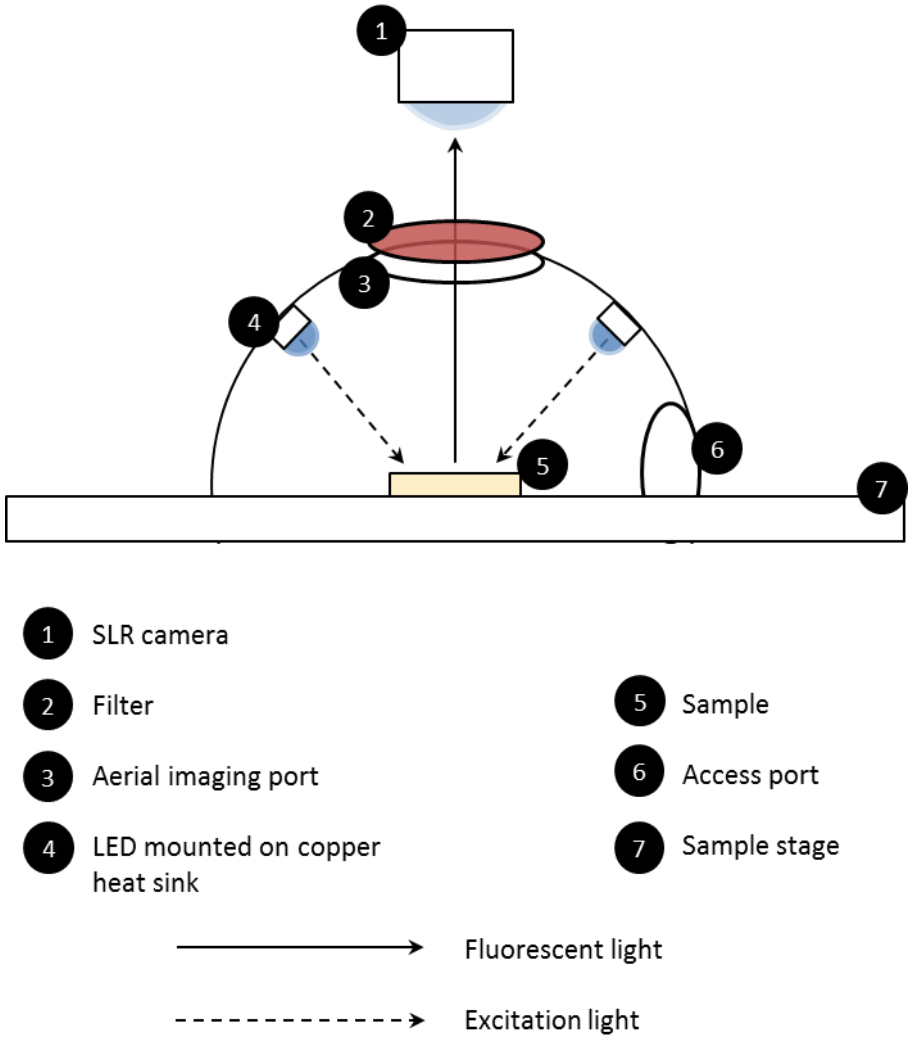


Figure 2.9. Schematic of QLF-*in vitro* system comprising modified lighting set-up for improved illumination of samples at 405 nm. Adapted from Hope *et al*, 2011.

2.8 ENAMEL SPECIMEN ANALYSES

2.8.1 Quantitative Light Induced Fluorescence (QLF): enamel imaging

Biofilm on enamel discs that were half painted with nail varnish was removed and discs were sterilised by thorough washing with 70% ethanol. The nail varnish was removed from the surface of the enamel using acetone to expose the sound tissue region and allowed to fully air-dry before being imaged using a custom-made setup incorporating the QLF™-D (QLF™-*in vitro*) or Bilumator (Inspektor Research Systems BV, Amsterdam, The Netherlands) technology, as described in section 2.7.4. In line with the methodology of the system, the sound tissue area was defined as a reference patch or control region and images were analysed to obtain ΔF values as an indication of mineral content. The distance between the camera and the surface of the enamel disc was standardised between experiments.

2.8.2 Non-Contact Surface Profilometry (NCSP)

Samples were scanned across a 3 mm by 1.5 mm area to include regions not exposed and exposed to experimental conditions, using NCSP (Proscan 2000 version 2.1.0, Scantron Industrial Products Ltd, Taunton, England, UK), S5/03 sensor-head (resolution 0.01 μm, measuring range 0.3 mm, spot size 4 μm) at a sample rate of 1000 Hz with an averaging (Kalman) filter of 2 iterations. Step size was 0.003 μm, selected by calculating optimum settings for capturing surface roughness in accordance to manufacturer advice, with 1000 steps on the X-axis and 500 on the Y-axis. Both varnish-protected and treatment-exposed sides of each sample were included in each scan and they took about 14 minutes to complete. When using Proscan for NCSP an optical confocal multiplexing sensor travels the programmed course of a sample measuring height deviations along its path. Figure 2.10 provides an overview of the chromatic sensor technology of the Proscan 2000. The sensor head is fixed in position, the sample is placed on a flat plate which moves the sample precisely in relation to

the sensor; linearly in the X and Y directions specified. White light is conducted through a lens with manufactured chromatic aberrations which enables it to split the white light into the full spectral field whilst focusing each frequency of colour at specific points within a defined measuring range. Once the sample is placed within this range, only one colour frequency is transmitted back from the surface. This light is fed back to a spectrometer that analyses the signal, converting it to a distance measurement. By combining the height data with information about the precise location of the moving table the three-dimensional profile is constructed. This can be analysed using dedicated tools within the Proscan 2000 software.

For this body of work surface roughness (in ISO Ra) and 2-point step-height for measuring surface loss were quantified. Following manufacturer advice all scans were firstly autolevelled within the Proscan 2000 software; this calculated the straight line of best fit to the data, subtracting it from the original data and displaying a new, level scan ready for further analysis. The "surface filter" tool was then applied to remove the general form of the profile leaving only the texture. The appropriate filter value to apply in this instance was calculated as "133" using the approach in Eqn 2.1. As advised, the filtered data was removed from the beginning and end of each scan line using the zoom function. According to ISO 4288-1996 the cut-off for data captured with the settings described above is 0.4 mm. The enamel surfaces were characterised in triplicate; regions were defined systematically along the Y-axis length of each scan as illustrated in Figure 2.11.

(cut-off filter in mm / 2) / step size in mm = surface filter

(0.8 mm / 2) / 0.003 mm = 133 (Eqn 2.1)

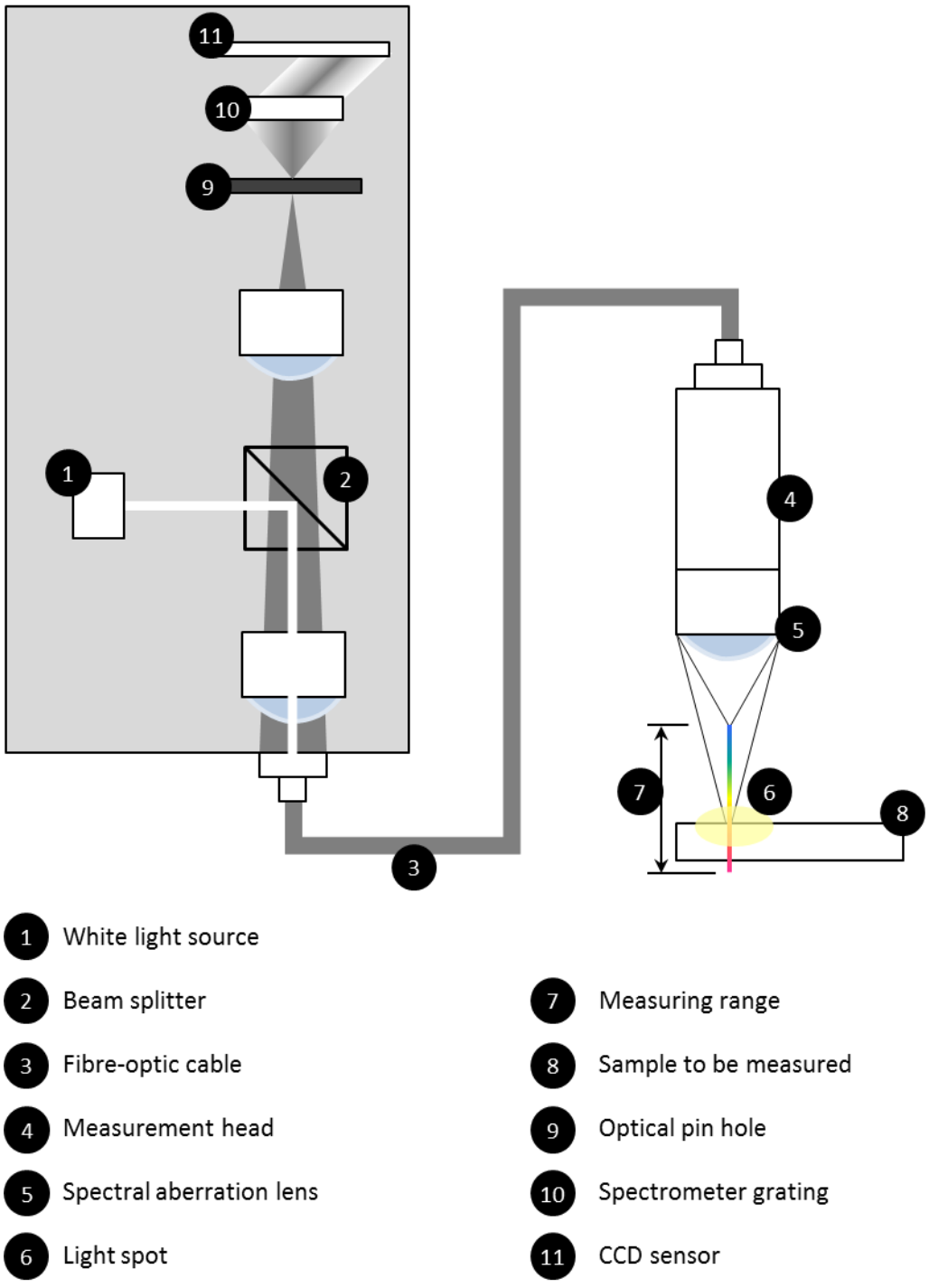


Figure 2.10. Principle behind chromatic sensor technology of Proscan 2000. Adapted from Scantron Ltd. manual

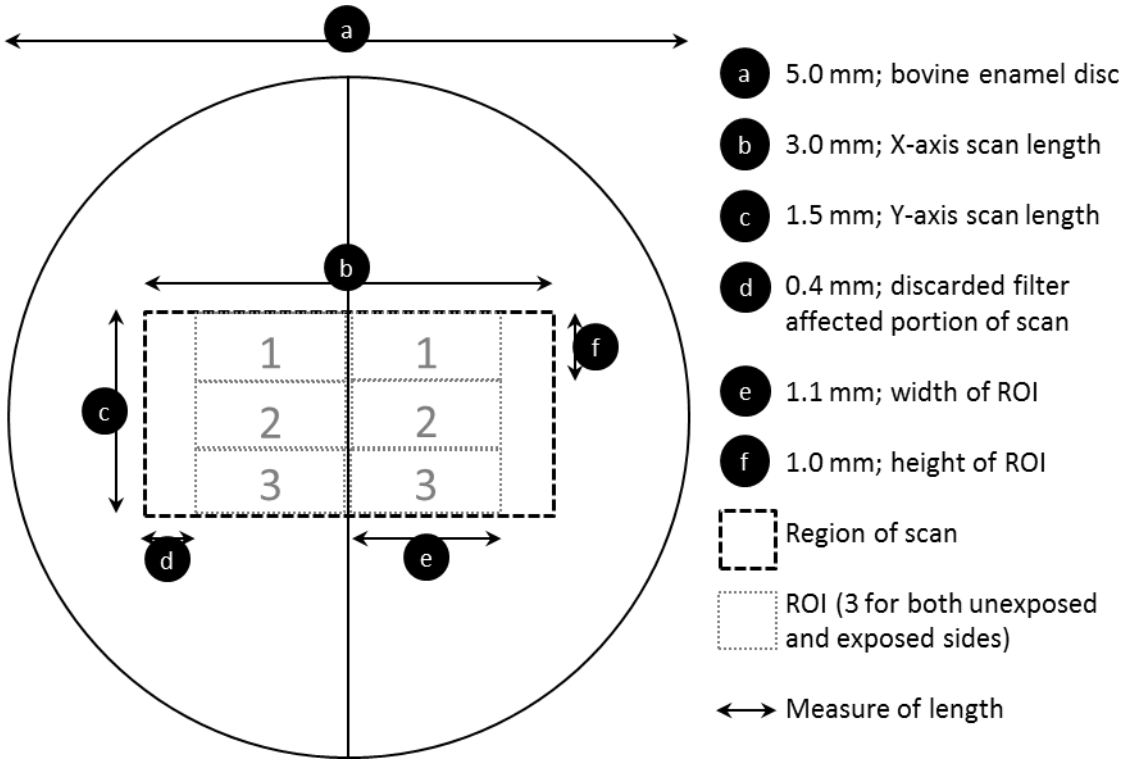


Figure 2.11. Schematic of a disc showing the specific area included in each NCSP scan (long dash) along with the three systematically defined regions of interest when measuring profile differences along each scan (small grey dash). Unexposed side on left and exposed side on right.

2.8.3 Scanning Electron Microscopy (SEM) & EDX

The surface of bovine enamel samples was scanned without prior coating using a Hitachi TM3000 desktop Scanning Electron Microscope (SEM; Hitachi High-technologies Europe GmbH., Berkshire, UK) up to x2000 magnification. Images were captured of the painted and unpainted half, to visualise the unexposed and exposed surfaces respectively, using the associated manufacturer software (Version 02.03.02). Images including the junction between both halves were also captured. Manufacturer software was also used to ascertain the elemental composition of selected regions of interest using energy dispersive X-ray spectroscopy (EDX) technology.

2.8.4 Transverse Microradiography (TMR)

Once all other analysis of the enamel specimens was complete they were prepared for microradiographic analysis to assess mineral loss (ΔZ) and lesion depth. Thin sections (approximately 1.2 mm) from each enamel disc were obtained using a water-cooled vertical diamond wire saw (Model 3242, Well Diamond Wire Saws Inc., Walter EBNER, Le Locle, Switzerland) and mounted on brass anvils using nail varnish and allowed to harden overnight. These sections were manually ground planar-parallel, first on one side to a thickness of 250 μm before being remounted and ground on the other to a final thickness of 80 μm using a custom-made, water-lubricated diamond disc (15 μm particle size; Buehler, Illinois, USA). Sections were analysed with transverse microradiography (TMR) as described previously (de Josselin de Jong *et al.*, 1987). They were mounted onto an acetate template and placed on a high resolution microradiographic plate (Kodak type 1A High-Resolution Plates, Kodak, Rochester, USA) with an aluminium step wedge (25 μm steps) for calibration and exposed to a CuKα X-ray source at 10 mA and 30 kV for 35 minutes. Plates were developed in accordance with manufacturer's instruction and solutions (Kodak D-19 Professional Developer, Kodak, Rochester, UK and Kodak Unifix, Kodak, Rochester, UK). Drawing on the formula of (Angmar *et al.*, 1963) for calculating mineral content using densitometric values; mineral content depth profiles were obtained using a Leica Leitz DMRB optical microscope (Leica, Wetzlar, Germany) densitometer-computer set-up inclusive of a Sony XC-75CE CCD camera (Sony, Tokyo, Japan) and TMR 2000 version 2.0.27.18 software (Inspektor Research Systems BV, Amsterdam, The Netherlands). Images were captured at a magnification of x20. Lesion variables such as integrated mineral loss (ΔZ) and maximum lesion depth were quantified using TMR 2006 (Version 3.0.0.10, Inspektor Research Systems BV, Amsterdam, The Netherlands).

2.9 STATISTICAL ANALYSES

All statistical analyses were implemented using SPSS for Windows Release 16.0.2 (SPSS UK Limited, Woking, UK) and Microsoft Excel 2010 (Version 14.0.7116.5000; Microsoft Office Professional 2010; Microsoft Corporation, Redmond, WA, USA). In order to apply parametric tests (where it is assumed that data is normally distributed) viable counts per population of microorganisms were subjected to $\log_{10}$ transformations. The logarithms ($\log_{10}[x+1]$, where x = surface loss, μm) of surface loss data were also obtained to satisfy assumptions of normal distribution. Data were subjected to routine Grubb's iterative tests ($\alpha=0.05$) for outliers and no values fell within this range in all data sets. A more explicit account of the statistical analyses chosen for each investigation is included with its associated experimental procedure. The threshold for statistical significance was defined as $p < 0.05$.

Chapter 3 Developing a biologically relevant caries model

3.1 INTRODUCTION

An *in vitro* caries model capable of taking into account biologically relevant parameters whilst allowing the simultaneous study of both the microbial biofilm and the caries lesion in dental enamel has not yet been developed. Dental caries has been studied extensively using abiotic model systems, yet these do not fully represent the mix of acids and bases produced by the diverse community of oral biofilm, nor their co-operative metabolism of dietary substrates. Though examining biofilm in its natural location is the most direct way of investigating it, natural systems are often difficult to deal with, expensive, and face significant obstacles with respect to reproducibility. A number of *in situ* model systems have been developed to assess enamel caries (Zero, 1995; Gameiro *et al.*, 2009) and dentine caries (Lima *et al.*, 2008), nevertheless, such approaches are unsuitable for testing experimental products for fear of subject safety, and cannot be used effectively in primary screening processes since they permit only a small number of replicates. *In vitro* models of dental plaque biofilm need to be able to imitate the complex environment they exist within. Laboratory models allow for the more precise and flexible control of environmental parameters; so results are more likely to be reproducible than those derived from *in situ* models. Furthermore, it has been recognised that *in vitro* models have provided information of far greater quality than *in situ* models or clinical trials (NIH, 2001), therefore making key contributions to the major advances observed in dental research.

Ideally an effective model system for studies in dental caries and oral biofilm should:

- incorporate dental hard tissue substratum
- provide sufficient biomass accumulation of biologically representative bacteria whilst also precluding the formation of too much biomass so biofilm on a whole is more representative of the *in vivo* situation
- allow the study of microcosm, defined consortia or of pure culture inoculates
- generate large quantities of samples so that the statistical reproducibility of experiments can be examined
- enable aseptic sampling procedures and be sterilised easily
- samples should be removable with biofilm intact on its corresponding substrata so it is disturbed as little as possible; hence maintaining the spatial organisation of the film
- enable the sequential removal of samples without affecting biofilm remaining in the system for subsequent sampling
- allow long-term experiments, intermitted de- and remineralisation cycles and different treatments within one experiment
- be sufficiently reproducible within and between experiments.
- mimic the nutrient flow characteristics of saliva by possessing a low medium volume: substratum surface ratio

The constant depth film fermenter (CDFF) attempts to satisfy these criteria. To date, it has been used to investigate biofilm structure and bacterial vitality in supragingival (Hope and Wilson, 2003) and subgingival (Hope and Wilson, 2006) microcosms; to study the efficacy of antimicrobials on biofilm (Kinniment *et al.*, 1996a; Pratten *et al.*, 1998a; Pratten *et al.*, 1998b; Pratten and Wilson, 1999; Pratten *et al.*, 2000) and to study the remineralisation (Deng *et al.*, 2005) and demineralisation of dentine by single species model biofilm (Deng and ten Cate, 2004). So far there is no evidence that the CDFF has been used to develop a model that studies enamel demineralisation alongside aspects of a biologically relevant biofilm.

3.1.1. Sugars and dental caries

The specific role of sugar consumption in dental caries has garnered extensive debate. Nonetheless, general consensus is that its role is, and has been, important. Historically, the most significant increases in the prevalence of caries have been associated with dietary changes (Suddick and Harris, 1990) and the converse is also true. For example, decay rates declined significantly when dietary sucrose was restricted during World War II among European (Toverud, 1957) and Japanese (Akizawa *et al.*, 1990) populations. As early as the 1950's, the Vipeholm Study demonstrated that the more frequently sugar is consumed, the greater the caries risk and that sugars consumed between meals possessed greater caries potential than those consumed during a meal (Gustafsson *et al.*, 1954), while the relationship between sugar exposure and the acidification of dental plaque and caries incidence was elucidated even earlier (Stephan, 1940; Stephan, 1944). More recently a strong correlation between caries incidence and national sucrose availability over a 50 year period was observed in an extensive national survey of the UK (Downer, 1999), and in Japan (Miyazaki and Morimoto, 1996). Whereas a linear relationship between caries experience and sugar consumption was reported when collectively analysing data from 90 countries yet individual analysis showed no such relationship in 29 industrialised nations (Woodward and Walker, 1994). This is likely due to the influence of the widespread use of fluoride dramatically decreasing caries prevalence despite the consumption of high levels of sugar. It is also traditionally difficult to obtain reliable data on the levels of sugar intake (Sreebny, 1982; Honkala and Tala, 1987; Marthaler, 1990). Despite debate, a majority of opinion based on evidence from human studies; as well as indirect evidence from laboratory studies (Renz and Bibby, 1989), *in situ* studies (Brudevold *et al.*, 1988; Kashket *et al.*, 1994; Lingstrom *et al.*, 1994; Pollard, 1995) and animal studies (Shaw, 1983) supports the relationship between sugar consumption and caries experience. It was posited that frequency of sugar consumption is of greater importance than total sugar consumption and that

sugars, particularly sucrose, are the most important dietary cause of caries (Sheiham, 2001; Zero, 2004). The intake of extrinsic sugars greater than 4 times a day appear to increase caries risk and here the author recommends that sugar consumption should not exceed 60 g/day for teenagers and adults and proportionately less for younger children.

Sucrose in the diet is a key environmental factor influencing changes in the composition of the biofilm matrix which in turn are strongly associated with the transition from health to disease. It is widely regarded as the most cariogenic carbohydrate since sucrose is fermented by cariogenic bacteria, producing a host of acids, serves as a key substrate for the synthesis of intracellular polysaccharide (IPS) and is the sole substrate for the synthesis of extracellular polysaccharide (EPS) (Rolla, 1989; Bowen, 2002; Paes Leme *et al.*, 2006). The abilities to produce both polysaccharide stores are crucial cariogenic features of dental plaque bacteria as reviewed earlier (Section 1.4.3.4). *S. mutans* dental plaque derived from sucrose-exposed cultures was found to be significantly more cariogenic than glucose and fructose-exposed plaque in an intraoral model (Zero *et al.*, 1986b; Cury *et al.*, 2000). In an *in situ* study, higher enamel mineral loss was observed with sucrose than with mixtures of equimolar concentrations of fructose and glucose (Cury *et al.*, 2000). Another study demonstrated that mineral loss and counts of mutans streptococci in the biofilm were higher, and accompanied by lower plaque pH values, in sucrose exposed biofilm *in situ* compared to starch exposed alone (Ribeiro *et al.*, 2005). Here, sucrose enhanced the cariogenic potential of starch when exposed in combination. Biochemical changes in the composition of the extracellular matrix of dental plaque-like biofilm were promoted by this carbohydrate (Cury *et al.*, 2000) and are dependent on frequency of sucrose exposure (Ccahuana-Vásquez *et al.*, 2007) and sucrose concentration (Aires *et al.*, 2006) in particular. Frequency of exposure and concentration of sucrose appear clearly associated to its cariogenicity (Konig *et al.*,

1968; Hefti and Schmid, 1979; Cury *et al.*, 1997; Duggal *et al.*, 2001; Paes Leme *et al.*, 2004; Aires *et al.*, 2006). Increased frequency leads to prolonged periods within the dental plaque where it is below critical pH for enamel demineralisation, whilst greater decreases in pH are observed as sucrose concentration is increased. Greater cariogenicity has been attributed to the sucrose-mediated synthesis of glucans which increases the porosity of plaque; allowing for greater biofilm penetration by the sugars of the diet and a rise in acidity directly at the tooth surface (Dibdin and Shellis, 1988; van Houte *et al.*, 1989). Additionally, sucrose disrupts the balance of the dental plaque microbial community, favouring the growth of cariogenic species (Marsh, 1991). It has been shown that sucrose promotes an increase in the proportions of mutans streptococci and *Lactobacillus* spp., whilst a fall in the numbers of *S. sanguinis* is observed due to the fall in pH associated with sucrose fermentation (de Stoppelaar *et al.*, 1970; Dennis *et al.*, 1975; Minah *et al.*, 1981). Higher mutans streptococci and *Lactobacillus* spp. counts were also reported in other studies, whilst demonstrating that biofilms formed in the presence of sucrose had lower fasting pH and final pH levels compared to those grown in the absence of this carbohydrate (Zero *et al.*, 1986a; Ribeiro *et al.*, 2005).

Aims

This study aimed to develop a biologically relevant caries model and investigate the process of caries lesion formation within it. It seeks to explore the *in vitro* application of the QLF™D system to assess changes in mineral content against TMR whilst investigating the changes in microbial dynamics within a multispecies biofilm in response to high frequency sucrose pulsing of different concentrations. Related work aimed to monitor the growth of the saliva pool organisms during typical CDFF inoculation.

3.2 EXPERIMENTAL PROCEDURE

3.2.1. Investigating microbial & pH dynamics during CDFF inoculation

The standard operating procedure when inoculating a CDFF involves the inoculation of mucin-containing artificial saliva growth medium which is delivered onto samples at a constant flow rate. One litre of artificial saliva growth media was produced as outlined in Section 2.6 and inoculated with an aliquot of the saliva pool (Section 2.2). Inoculated medium was pumped out of the vessel at a 0.72 mL min^{-1} flow rate, through tubing usually connected to a CDFF, but instead diverted into a sterilised bottle for later discard. This experimental design mimicked the inoculation of a CDFF over the traditional twenty four hour inoculation period. Samples were intercepted hourly by collecting droplets of media destined for the effluent jar in sterile plastic bijous. Microbial composition was enumerated as described in Section 2.7.1 for total viable bacteria, total *Streptococcus* spp., total mutans streptococci, black pigmented anaerobes and sulfur producers. pH of each sample was also measured and the experiment was undertaken on two separate occasions.

3.2.2. Effect of sucrose concentration within a CDFF caries model

The experimental procedure to produce caries lesions in the CDFF model is illustrated in Figure 3.1. The CDFF without sample pans was sterilised by dry-heat whereas the rest of the associated apparatus was sterilised by autoclave as described in Section 2.5. Three, half-painted bovine enamel discs and two hydroxyapatite discs were loaded into each CDFF sample pan and recessed to a depth of 200 μm prior to sterilisation by gamma irradiation. Loaded sample pans were introduced into the CDFF turntables aseptically within a laminar flow cabinet and biofilm was produced as described in Section 2.6.

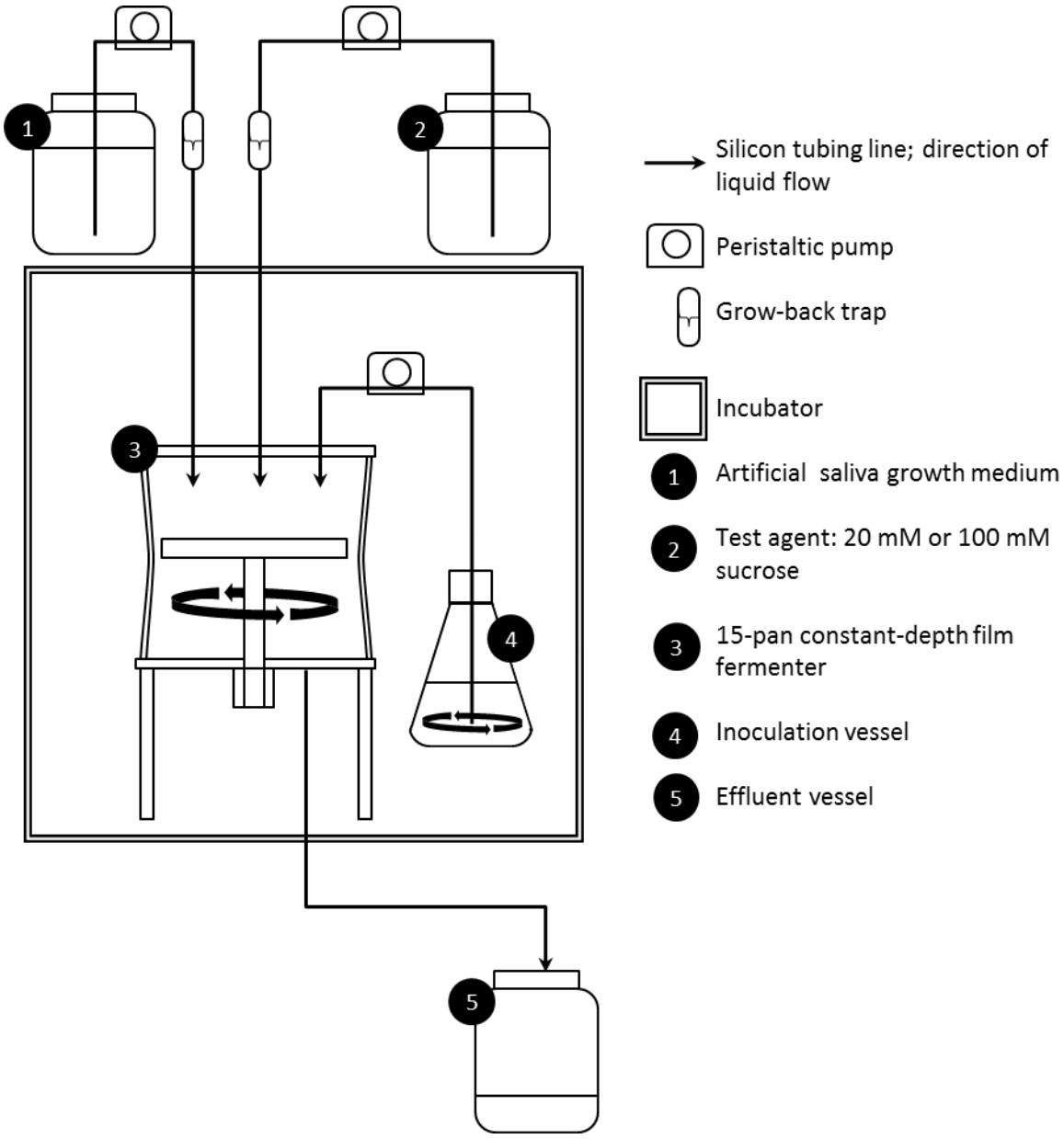


Figure 3.1. Circuit diagram with key of experimental procedure to investigate the effect of sucrose concentration within a constant-depth film fermenter caries-biofilm model.

Twenty two mM & 110 mM sucrose (Sigma-Aldrich, Poole, UK) solutions were prepared and autoclaved at 116°C (1900 mBar) for 15 minutes to prevent caramelisation of the dissolved sugars. These stock concentrations were calculated to take into account the dilution effect of the artificial saliva growth media pumping simultaneously so that the final concentration within the CDFF of each sucrose-pulsed experiment was 20 mM and 100 mM respectively. The CDFFs were pulsed at

a flow rate of 5 mL min^{-1} via a timer controlled peristaltic pump every 2 hours 8 times daily for 15 mins with a 10 hour rest; to mimic the typical lack of sucrose consumption at night. Sucrose flow was first initiated 18 hours after inoculation began (8 hours after sterile artificial saliva growth media began). The experiment was repeated without sucrose addition to serve as a control.

Substrata samples laden with biofilm were extracted at day 4, 6, 8, 10, 12 and 14 in each experiment. Sample pans were removed and photographed with biofilm intact using QLF-D for subsequent analysis of biofilm fluorescence (see Section 2.7.4). All discs were then extracted from the sample pans using sterile forceps. Enamel discs were sterilised in 70% ethanol and reserved for enamel specimen analysis whilst the two hydroxyapatite discs laden with biofilm were vortex mixed for 30 seconds in 900 μL of phosphate-buffered saline (PBS) to disrupt the biofilm and the resulting cell suspension was serially diluted up to 10^6 in additional PBS. Selective solid agar was used to isolate and enumerate total viable bacteria, total *Streptococcus* spp, total mutans streptococci group and *Lactobacillus* spp. as described in Section 2.7.1. Enamel discs half-painted with nail varnish were photographed with QLF and analysed for fluorescence loss (see Section 2.8.1), scanned for surface changes using NCSP (see Section 2.8.2) and finally subjected to TMR methodology to elucidate any sub-surface changes in mineral content as described in Section 2.8.4. Extra sample pans were extracted and used to investigate biofilm pH changes in response to the addition of sucrose. A micro pH meter was set up so that its probe was in contact with the biofilm but elevated so that it was not in direct contact with the substratum. 20 μL saline, 20 mM or 100 mM sucrose were pipetted onto individual biofilms and the pH was recorded over time.

The effect of 100 mM sucrose on enamel demineralisation was modelled in a repeat experiment in order to properly address the aim of investigating the concordance between QLF and TMR. This was required since it was suspected that correlations between these methods were influenced by problems that arose in preparing enamel sections for TMR in the previous study.

3.2.3. Statistical analyses

The hypothesis that there are differences in enamel mineral content (as assessed by QLF and TMR) and in surface changes (quantified by NCSP) when exposed to microcosm biofilm subjected to different sucrose concentrations was tested using single factor ANOVA and two-way ANOVA analyses. Tukey's or Sidak's multiple comparisons tests were used post-hoc to identify the statistical significance of differences between outcomes (a) at each time within a given experiment and (b) for differences between each experiment for each time point. An analysis of covariance (ANCOVA) model able to adjust for time was used to test the hypothesis that sucrose affects the growth of microbial constituents of the biofilm. Pearson's product moment coefficients (r) were calculated to determine how well QLF and TMR correlate in the assessment of caries. Prior to all analysis normal distributions of the raw data was checked and transformed where needed and all datasets were routinely checked for outliers as described in Section 2.9.

3.3 RESULTS

3.3.1 Microcosm growth & pH dynamics in CDFF inoculation vessel

Results (mean ± SD) from two different experiments showed that after an initial lag phase, logarithmic growth of total bacteria was observed before reaching stationary phase at 8 hours (Figure 3.2). This initial increase in the total number of bacteria peaked by around the eighth hour ($4.48 \times 10^8 \pm 1.78 \times 10^8$ cfu mm^{-2} in exp.1 and $3.94 \times 10^7 \pm 6.38 \times 10^6$ cfu mm^{-2} in exp. 2 respectively) whilst the numbers of mutans streptococci peaked and decreased after the tenth (3.70

x $10^4 \pm 3.04 \times 10^4$ cfu mm^{-2}) and eleventh hour ($3.01 \times 10^3 \pm 1.25 \times 10^3$ cfu mm^{-2}) until the end of experiment 1 and 2 respectively. Sulfur producers and black-pigmented organisms were not well supported. pH fluctuated early on before coming to a plateau, corresponding to the changes in the microbial populations reported.

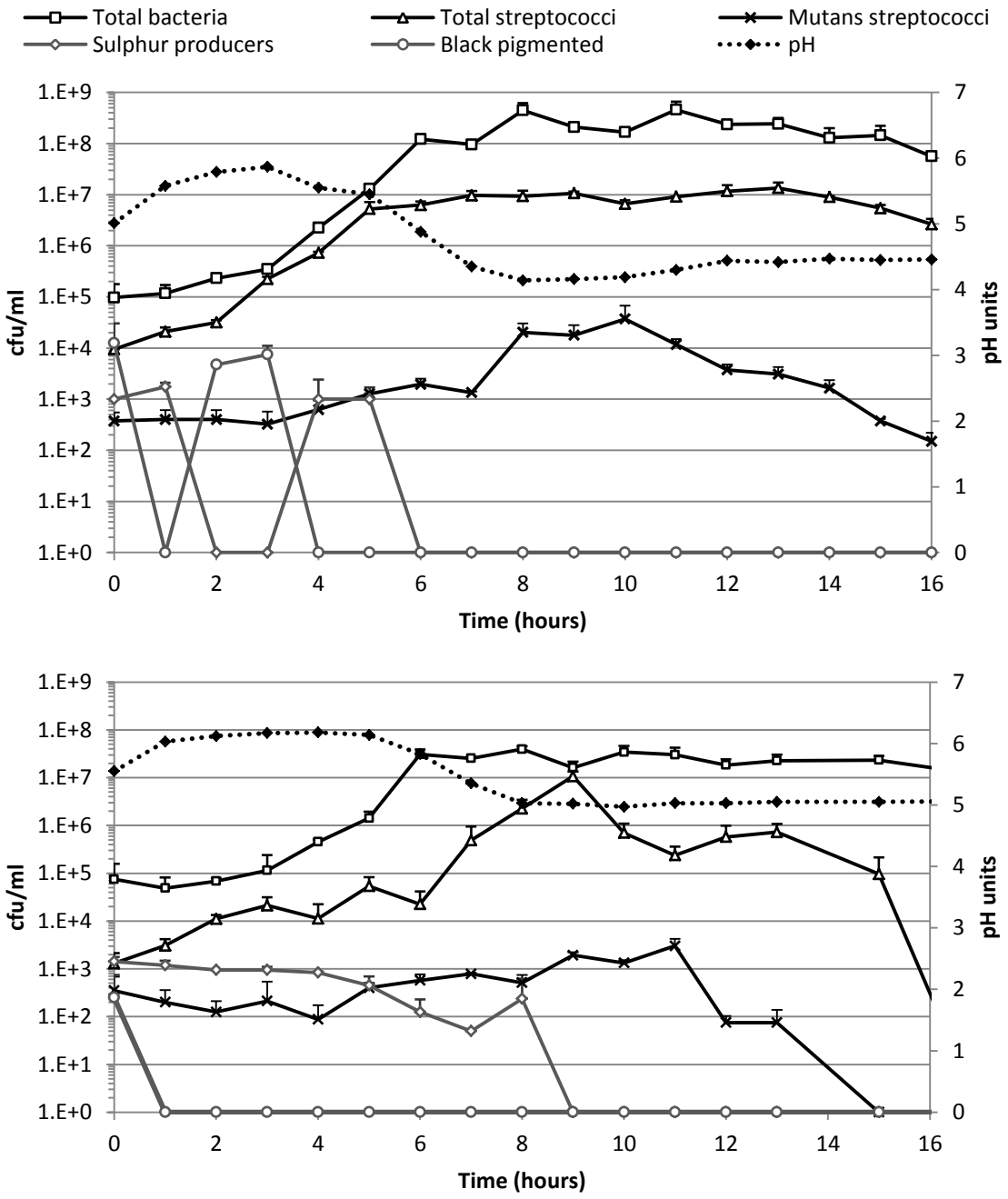


Figure 3.2. Growth of microcosm saliva pool bacteria and changes in pH of inoculated artificial saliva growth medium in CDFF inoculation vessel. Results from replicate experiments are included (above and below). Error bars represent standard deviation, $n = 4$.

3.3.2 Effect of sucrose concentration on enamel demineralisation

Both QLF fluorescence change (ΔF, %) and mineral loss (ΔZ, %Vol.μm) measured by TMR analysis revealed that pulsing with 100 mM sucrose provided a quicker, more pronounced caries progression pattern than pulsing with 20 mM sucrose (Table 3.1 and Figures 3.3 – 3.5). When comparing fluorescence changes between experiments, differences were statistically significant between 100 mM sucrose exposed samples and the control group only at day 10, 12 and 14 ($p = 0.0002$, $p < 0.0001$ and $p < 0.0001$ respectively) and between 100 mM and 20 mM sucrose on day 12 and 14 (Figure 3.5a). The difference between fluorescence changes in 20 mM exposed samples and samples of the control group was found to be statistically significant only on day 14 ($p = 0.0004$). When comparing mineral loss and lesion depth between experiments (Figure 3.5b and Table 3.1), statistically significant differences were found between observations in day 12 and 14 samples exposed to 20 or 100 mM sucrose respectively ($p < 0.0001$ for all). However, differences were found to be significant for samples exposed to 20 or 100 mM sucrose at day 6 also ($p < 0.0001$). Some fluorescence loss was observed in the enamel substrata of biofilm exposed to no sucrose throughout the experiment but at generally lower levels than when biofilm was exposed to either concentration of sucrose. Differences over time were also found not to be statistically significant. No caries lesions were found in these samples when studied with TMR. At 20 mM sucrose a maximum loss of -16.57 ± 7.3 SD ($n = 3$) in ΔF was reached by day 14 with a generally minimal ΔF in preceding days. However, no statistically significant differences in ΔF, ΔZ and lesion depth were observed between time points at this concentration. At 100 mM sucrose a maximum change in ΔF of -52.60 ± 7.5 SD ($n = 3$) was observed by day 12 and, when compared to ΔF in day 14 samples, differences were found not to be statistically significant (Table 3.2). Differences were statistically significant in the fluorescence loss of samples of both these days compared to day 4, 6, 8 and 10 samples ($p < 0.05$). Furthermore, at 100 mM sucrose maximum ΔZ was detected by day 12 as 1972.18 ± 512.4 SD ($n = 3$) with lesion depth

of 69.78 ± 24.51 (n = 3) compared to a maximum of 225.69 ± 24.35 SD (n = 3) ΔZ and 9.80 ± 1.11 (n = 3) lesion depth in 20 mM exposed samples by day 14. This difference was statistically significant (Figure 3.5b and Table 3.1). As with QLF, TMR was able to resolve statistically significant differences of samples from day 12 and 14 compared to 4, 8 and 10 respectively ($p < 0.05$) but not at day 6 within 100 mM sucrose exposed samples, both in terms of mineral loss and lesion depth (Table 3.2). Converse to QLF ΔF observations, both ΔZ and lesion depth were found to be statistically significant between day 6 samples compared to samples of day 4, 8 and 10 respectively ($p < 0.05$).

Table 3.1. Lesion depth (μm) determined by TMR in bovine enamel samples exposed to multispecies oral biofilm pulsed with 20 mM or 100 mM 8 times daily. Data is for mean ± standard deviation, n = 3

Sucrose conc.	Lesion depth (μm) at given time (day)					
	4	6	8	10	12	14
20 mM	7.18 ± 3.05	**5.73 ± 2.86**	11.45 ± 6.42	9.88 ± 3.27	**9.80 ± 1.11**	**6.35 ± 2.88**
100 mM	12.24 ± 3.38	**57.06 ± 9.39**	18.82 ± 10.73	17.99 ± 8.54	**69.78 ± 24.51**	**62.74 ± 16.24**

bold font denotes difference is statistically significant when comparing between sucrose conc.

Figure 3.3. Representative QLF-D images of bovine enamel sampled longitudinally within a biological caries model subjected to 0 mM (control), 20 mM or 100 mM sucrose 8 times daily.

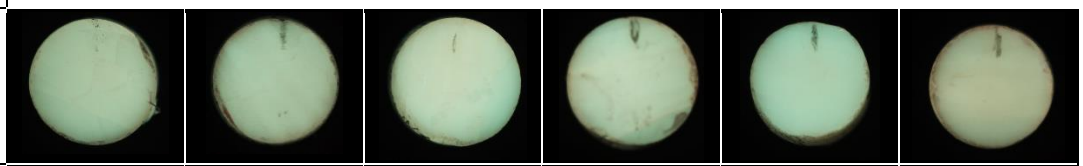
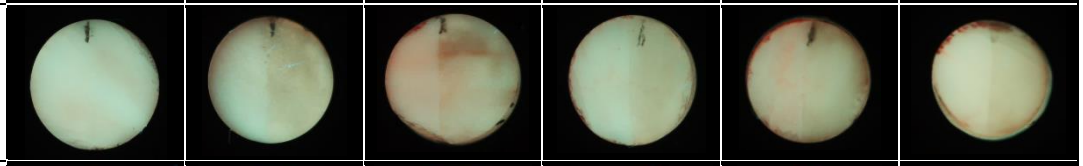
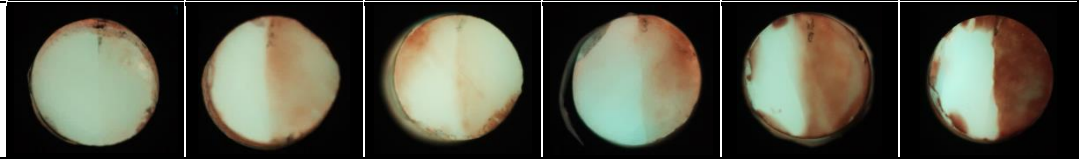
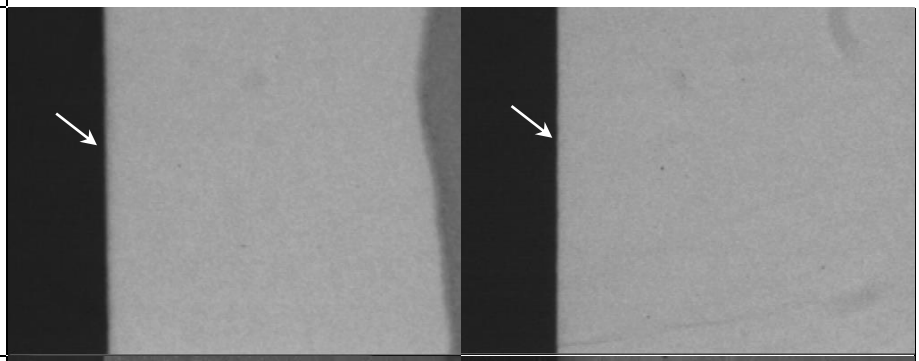
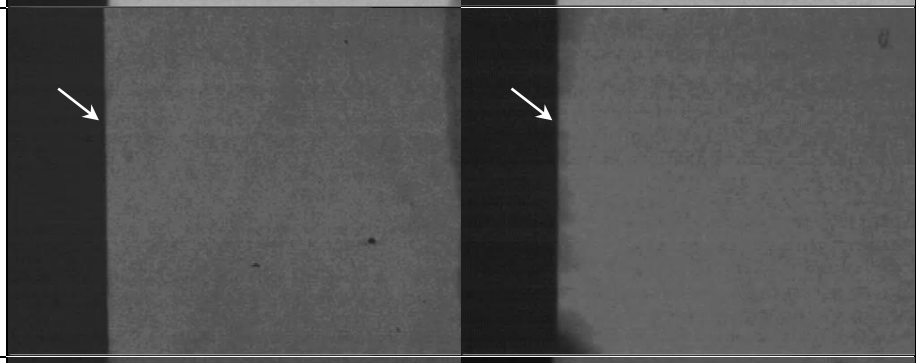
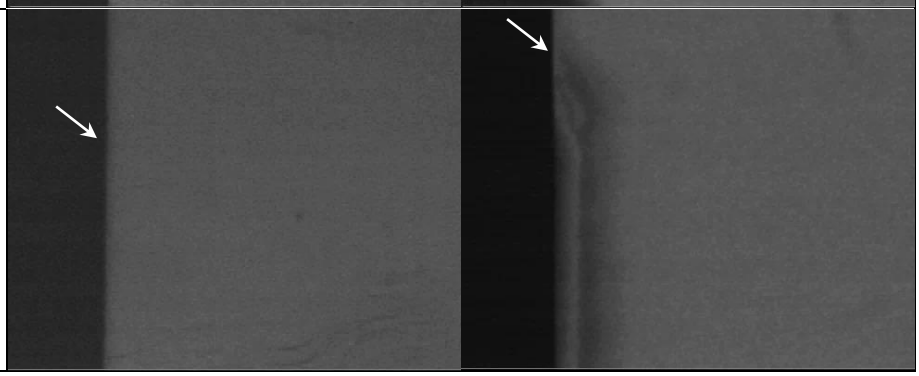
Sucrose conc.	Time (day)					
	4	6	8	10	12	14
0 mM						
20 mM						
100 mM						

Figure 3.4. Representative TMR images of bovine enamel sampled longitudinally within a biological caries model subjected to either no, 20 mM or 100 mM sucrose 8 times daily. Arrow indicates the interface between the biofilm exposed and nail-varnish protected zones.

Sucrose concentration	Time (day)	
	4	14
0 mM		
20 mM		
100 mM		

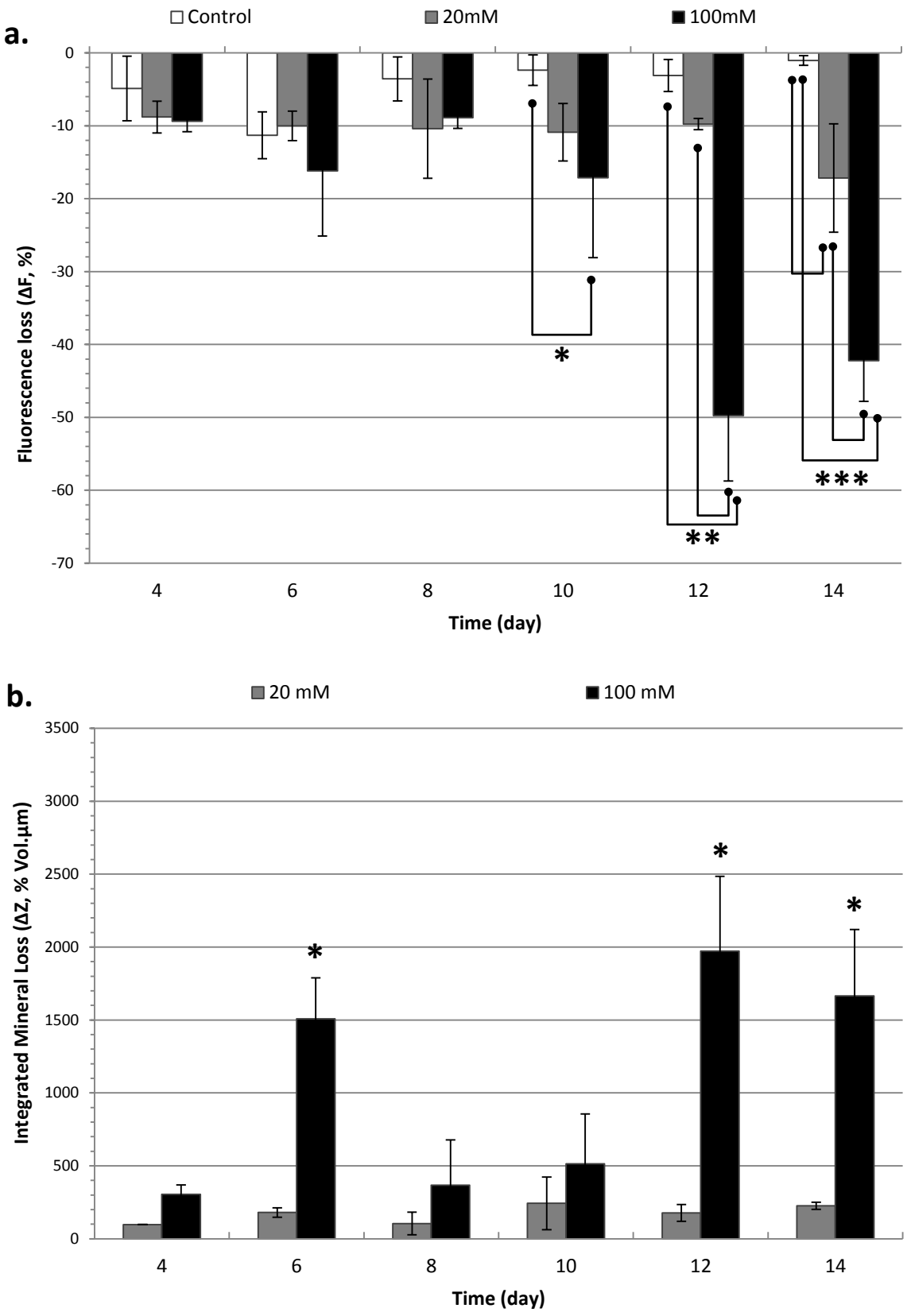


Figure 3.5. Longitudinal changes in (a) enamel fluorescence (average fluorescence loss, ΔF, %) as detected by QLF *in vitro* and (b) mineral loss (average ΔZ, %Vol.µm) determined by TMR in bovine enamel samples exposed to multispecies oral biofilm pulsed with 20 mM, 100 mM or no sucrose 8 times daily. Error bars represent standard deviation, $n = 3$. * means $p < 0.01$.

Table 3.2. Results of multiple comparisons testing the statistical significance between changes in enamel caries characteristics over 14 days in a caries model exposed to **100 mM** sucrose 8 times daily according to fluorescence change, mineral loss, lesion depth and surface loss (n = 3 throughout).

Time (day)	Fluorescence change (ΔF, %)			Mineral Loss *(ΔZ, %Vol.μm)*			Lesion Depth (μm)			Surface Loss* (μm)		
	MD	±CI$^{95\%}$	p	MD	±CI$^{95\%}$	p	MD	±CI$^{95\%}$	p	MD	±CI$^{95\%}$	p
4 vs 6	-9.29	-21.6, 3.00	0.226	-1203	-1888, -519	**0.0003**	44.8	19.8, 69.8	**0.0002**	-0.009	-0.063, 0.044	0.995
4 vs 8	-1.49	-13.8, 10.8	0.999	-62.8	-747, 621	0.999	6.58	-18.4, 31.6	0.959	-0.007	-0.061, 0.046	0.998
4 vs 10	-11.7	-24.0, 0.57	0.069	-210	-894, 475	0.925	5.75	-19.3, 30.8	0.977	-0.009	-0.063, 0.045	0.996
4 vs 12	-43.6	-55.9, 31.3	**< 0.001**	-1667	-2351, -983	**< 0.001**	57.5	32.5, 82.6	**< 0.001**	0.048	-0.006, 0.102	0.103
4 vs 14	-35.3	-47.6, -23.0	**< 0.001**	-1361	-2045, -677	**< 0.001**	43.3	18.3, 68.3	**0.0003**	-0.007	-0.061, 0.047	0.999
6 vs 8	7.80	-4.49, 20.1	0.405	1141	456, 1825	**0.0005**	-38.2	-63.3, -13.2	**0.0013**	0.002	-0.052, 0.055	> 0.999
6 vs 10	-2.43	-14.7, 9.86	0.990	994	310, 1678	**0.0022**	-39.1	-64.1, -14.0	**0.0010**	0.0003	-0.053, 0.054	> 0.999
6 vs 12	-34.3	-46.6, -22.0	**< 0.001**	-464	-1148, 220	0.312	12.7	-12.3, 37.7	0.609	0.0573	0.004, 0.111	**0.031**
6 vs 14	-26.0	-38.3, -13.7	**< 0.001**	-158	-842, 527	0.977	-1.54	-26.6, 23.5	> 0.999	0.0023	-0.051, 0.056	> 0.999
8 vs 10	-10.2	-22.5, 2.06	0.147	-147	-831, 537	0.983	-0.83	-25.9, 24.2	> 0.999	-0.001	-0.055, 0.0524	> 0.999
8 vs 12	-42.1	-54.4, -29.8	**< 0.001**	-1604	-2289, -920	**< 0.001**	51.0	25.9, 76.0	**< 0.001**	0.0557	0.002, 0.109	**0.039**
8 vs 14	-33.8	-46.1, -21.5	**< 0.001**	-1298	-1982, -614	**0.0001**	36.7	11.7, 61.7	**0.0020**	0.0007	-0.053, 0.054	> 0.999
10 vs 12	-31.9	-44.2, -19.6	**< 0.001**	-1458	-2142, -773	**< 0.001**	51.8	26.8, 76.8	**< 0.001**	0.057	0.003, 0.111	**0.032**
10 vs 14	-23.6	-35.9, -11.3	**< 0.001**	-1152	-1836, -467	**0.0004**	37.5	12.5, 62.5	**0.0016**	0.002	-0.052, 0.056	> 0.999
12 vs 14	8.27	-4.02, 20.6	0.342	306	-378, 990	0.723	-14.3	-39.3, 10.8	0.493	-0.055	-0.109, -0.001	**0.042**

Mean difference, MD; lower and upper confidence intervals, ±CI$^{95\%}$

*natural $\log_{10}$ transformed prior to analysis; **bold** font denotes difference is statistically significant

3.3.3 Effect of sucrose-fed biofilm on enamel surface loss

NCSP quantified nanometre scale surface changes (Figure 3.6) with trends similar to those observed by the QLF and TMR quantification of enamel demineralisation. Two-way ANOVA revealed that sucrose concentration was statistically significant ($p = 0.045$) but time and their interaction were not ($p = 0.204$ and 0.504 respectively). Less surface loss was observed in samples exposed to 20 mM sucrose than in those exposed to 100 mM sucrose except at day 8. The greatest surface loss was observed in enamel samples exposed to 100 mM sucrose for 12 days (0.175 ± 0.247 µm SD, $n = 3$ vs 0.040 ± 0.023 µm SD, $n = 3$ at 20 mM on day 12). No surface loss was detected in all enamel samples exposed to biofilm fed with no sucrose and differences observed between times of non-sucrose exposed, and in samples of the 20 mM group, were found not to be statistically significant following ANOVA and Tukey's multiple comparisons test. Tukey's test revealed that differences in surface loss between individual times in 100 mM exposed samples were significant between day 12 and 6 ($p = 0.031$), 12 and 8 ($p = 0.039$), 12 and 10 ($p = 0.032$), and 12 and 14 ($p = 0.042$) (Table 3.2). Comparing results between experiments, the differences in surface loss were found to be statistically significant only at day 12; wherein surface loss for 100 mM exposed samples statistically differed from control samples ($p = 0.0022$) and when compared to 20 mM sucrose exposed samples ($p = 0.0325$) at this time point (Figure 3.6). The NCSP was also able to resolve prism-like structures as mineral was lost from the enamel surface as a consequence of exposure to acids produced by bacteria of the biofilm (Figure 3.7).

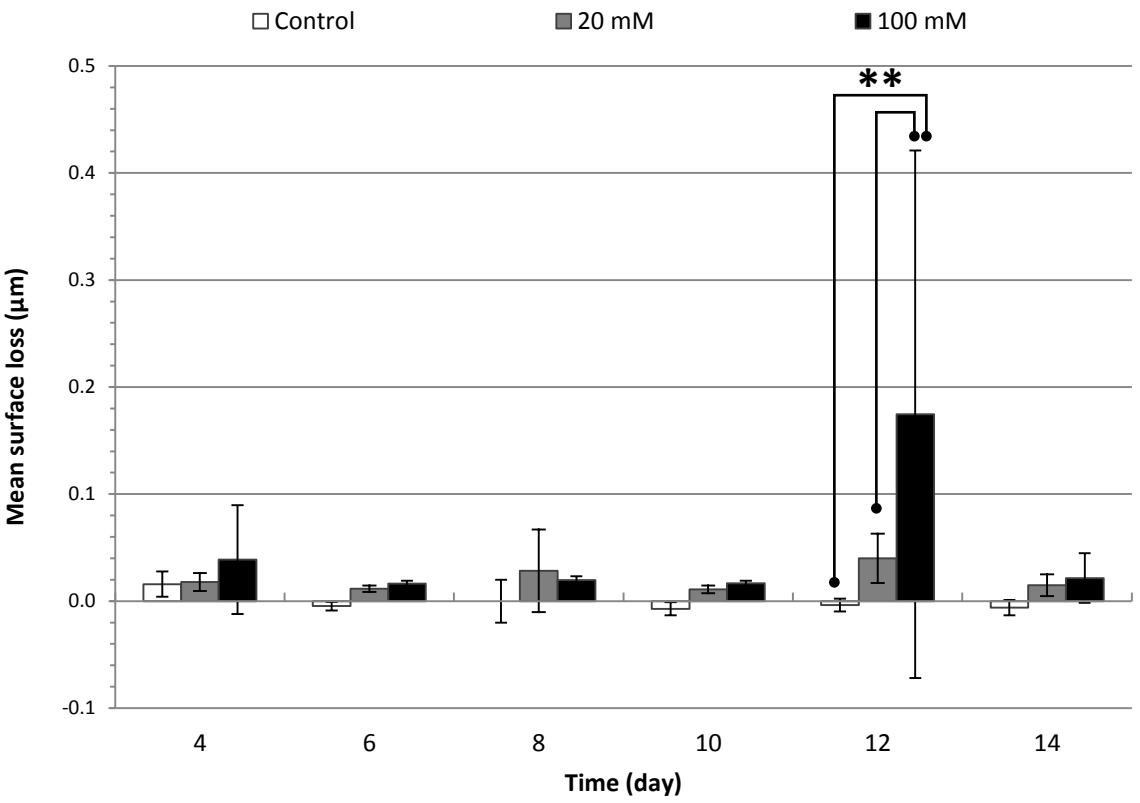


Figure 3.6. Mean surface loss (µm) of bovine enamel samples exposed to 20 mM, 100 mM or no sucrose 8 times daily as detected by NCSP measurements of height differences between negative control and treatment zones within samples. Error bars represent standard deviation, n = 3. * = $p < 0.001$.

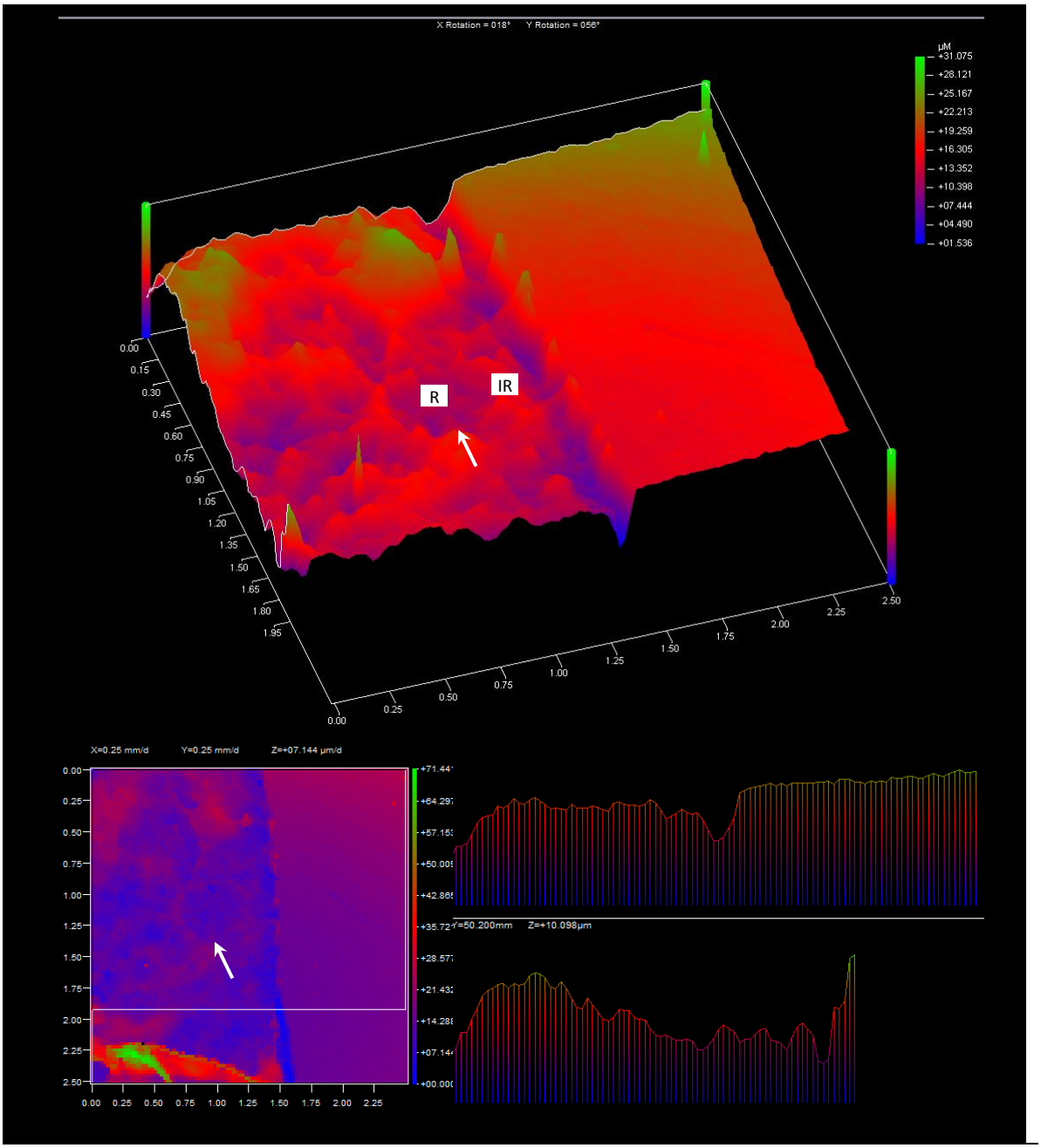


Figure 3.7. Example scan results of a bovine enamel sample exposed to cariogenic biofilm. Arrows indicate one of the prism structures apparent after surface loss as a result of exposure to bacterial acid from the biological caries model. Rod (R) and interrod (IR) enamel seem visible.

3.3.4 Longitudinal effects of sucrose concentration on microcosm biofilm development

Figure 3.8 shows the microbial composition of multispecies oral biofilm when pulsed with 20 mM or 100 mM sucrose 8 times daily or pulsed with no sucrose as a control. Counts of total viable bacteria showed a pseudo-steady state was achieved in all biofilm by day 4. Results (mean ± SD) indicate that at 20 mM sucrose exposure, mutans streptococci and *Lactobacillus* spp. achieved pseudo-steady state at $5.12 \times 10^6 \pm 3.70 \times 10^6$ cfu mm^{-2} and $6.20 \times 10^5 \pm 5.53 \times 10^5$ cfu mm^{-2} (average of day 8 – 14 counts, $n = 4$) respectively by day 8. At 100 mM *Lactobacillus* spp. also achieved steady state by day 8 (average $1.46 \times 10^6 \pm 2.59 \times 10^5$ cfu mm^{-2}, $n = 4$) whereas mutans streptococci growth was repressed; only achieving $3.36 \times 10^4 \pm 8.99 \times 10^3$ cfu mm^{-2} at day 8. In non-sucrose exposed biofilm mutans streptococci reached pseudo-steady state by day 8 (average $4.87 \times 10^4 \pm 3.44 \times 10^4$ cfu mm^{-2}, $n = 4$) while *Lactobacillus* spp. growth was limited; reaching only $1.08 \times 10^2 \pm 2.93$ cfu mm^{-2} ($n = 4$) by day 8. Differences in total bacteria, total *Streptococcus* spp., mutans streptococci and *Lactobacillus* spp. were statistically significant when comparing bacterial counts of biofilm not exposed to sucrose with 20 mM exposed biofilm (Table 3.3 – Table 3.6) and 100 mM exposed biofilm (Table 3.7 – Table 3.10) as well as when comparing 20 mM biofilm with 100 mM pulsed biofilm (Table 3.11 – Table 3.14). The effect of time and the interaction between sucrose and time was statistically significant for *Lactobacillus* spp. in all comparisons (Table 3.6, 3.10 and 3.14) whereas differences over time and its interaction with sucrose in mutans streptococci was significant only when comparing 100 mM sucrose exposed biofilm with non-pulsed control and 20 mM sucrose biofilm (Table 3.9 and Table 3.13 respectively). Differences were not statistically significant over time for mutans streptococci exposed to 20 mM sucrose pulsed compared to in the absence of sucrose (Table 3.5). Significant interactions suggest bacterial counts change differently at each time point when exposed to different concentrations of sucrose.

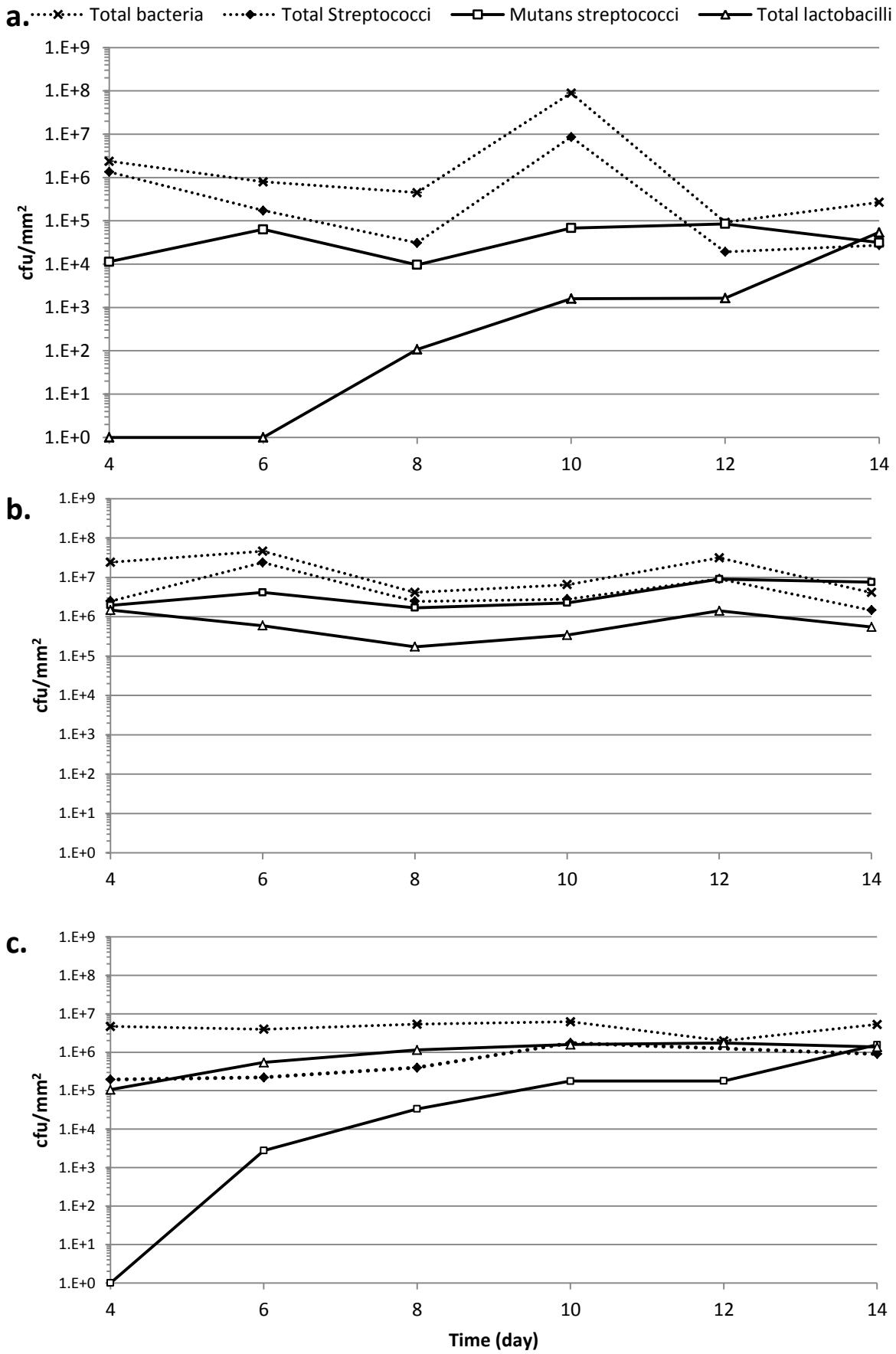


Figure 3.8. Microbial composition of multispecies oral biofilm grown on hydroxyapatite pulsed with (a) 0 mM, (b) 20 mM or (c) 100 mM sucrose eight times daily. Error bars represent standard deviation, $n = 4$.

Table 3.3. Results of ANCOVA testing statistical significance of time and sucrose concentration in **total viable bacteria*** exposed to **no or 20 mM sucrose**.

Source of Variation	SSIII	*df*	MS	F	*p*
Sucrose	308.122	2	154.061	266.916	**< 0.001**
Time	2.296	1	2.296	3.978	0.052
Sucrose x time	0.027	1	0.027	0.046	0.831

R Squared = 0.988 (Adjusted R Squared = 0.987)

Table 3.4. Results of ANCOVA testing statistical significance of time and sucrose concentration in **total *Streptococcus* spp.*** exposed to **no or 20 mM sucrose**.

Source of Variation	SSIII	*df*	MS	F	*p*
Sucrose	268.295	2	134.148	237.992	**< 0.001**
Time	4.029	1	4.029	7.148	**0.010**
Sucrose x time	1.644	1	1.644	2.916	0.095

R Squared = 0.986 (Adjusted R Squared = 0.984)

Table 3.5. Results of ANCOVA testing statistical significance of time and sucrose concentration in **mutans streptococci*** exposed to **no or 20 mM sucrose**.

Source of Variation	SSIII	*df*	MS	F	*p*
Sucrose	145.817	2	72.909	71.994	**< 0.001**
Time	2.072	1	2.072	2.046	0.160
Sucrose x time	0.037	1	0.037	0.037	0.849

R Squared = 0.970 (Adjusted R Squared = 0.967)

Table 3.6. Results of ANCOVA testing statistical significance of time and sucrose concentration in **total *Lactobacillus* spp.*** exposed to **no or 20 mM sucrose**.

Source of Variation	SSIII	*df*	MS	F	*p*
Sucrose	118.893	2	59.447	346.299	**< 0.001**
Time	32.424	1	32.424	188.883	**< 0.001**
Sucrose x time	35.577	1	35.577	207.251	**< 0.001**

R Squared = 0.992 (Adjusted R Squared = 0.992)

Type III sum of squares, SSIII; degrees of freedom, *df*; mean square, MS; F = (MS between groups)/(MS within groups). *$\log_{10}$ transformed prior to analysis. **bold** font denotes difference is statistically significant.

Table 3.7. Results of ANCOVA testing statistical significance of time and sucrose concentration in **total viable bacteria*** exposed to **no or 100 mM sucrose**.

Source of Variation	SSIII	*df*	MS	F	*p*
Sucrose	269.278	2	134.639	265.394	**< 0.001**
Time	0.864	1	0.864	1.704	0.199
Sucrose x time	0.561	1	0.561	1.105	0.299

R Squared = 0.989 (Adjusted R Squared = 0.987)

Table 3.8. Results of ANCOVA testing statistical significance of time and sucrose concentration in **total *Streptococcus* spp.*** exposed to **no or 100 mM sucrose**.

Source of Variation	SSIII	*df*	MS	F	*p*
Sucrose	243.373	2	121.687	43.717	**< 0.001**
Time	13.383	1	13.383	4.808	**0.034**
Sucrose x time	0.136	1	0.136	0.049	0.826

R Squared = 0.907 (Adjusted R Squared = 0.899)

Table 3.9. Results of ANCOVA testing statistical significance of time and sucrose concentration in **mutans streptococci*** exposed to **no or 100 mM sucrose**.

Source of Variation	SSIII	*df*	MS	F	*p*
Sucrose	61.033	2	30.517	40.228	**< 0.001**
Time	65.411	1	65.411	86.226	**< 0.001**
Sucrose x time	46.801	1	46.801	61.694	**< 0.001**

R Squared = 0.965 (Adjusted R Squared = 0.962)

Table 3.10. Results of ANCOVA testing statistical significance of time and sucrose concentration in **total *Lactobacillus* spp.*** exposed to **no or 100 mM sucrose**.

Source of Variation	SSIII	*df*	MS	F	*p*
Sucrose	89.392	2	44.696	312.726	**< 0.001**
Time	49.718	1	49.718	347.867	**< 0.001**
Sucrose x time	21.232	1	21.232	148.553	**< 0.001**

R Squared = 0.994 (Adjusted R Squared = 0.993)

Type III sum of squares, SSIII; degrees of freedom, *df*; mean square, MS; F = (MS between groups)/(MS within groups). *$\log_{10}$ transformed prior to analysis. **bold** font denotes difference is statistically significant.

Table 3.11. Results of ANCOVA testing statistical significance of time and sucrose concentration in **total viable bacteria*** exposed to **20 or 100 mM sucrose**.

Source of Variation	SSIII	*df*	MS	F	*p*
Sucrose	309.685	2	154.843	1448.746	**< 0.001**
Time	0.588	1	0.588	5.498	**0.024**
Sucrose x time	0.343	1	0.343	3.210	0.080

R Squared = 0.998 (Adjusted R Squared = 0.998)

Table 3.12. Results of ANCOVA testing statistical significance of time and sucrose concentration in **total *Streptococcus* spp.*** exposed to **20 or 100 mM sucrose**.

Source of Variation	SSIII	*df*	MS	F	*p*
Sucrose	260.570	2	130.285	54.298	**< 0.001**
Time	5.646	1	5.646	2.353	0.132
Sucrose x time	2.726	1	2.726	1.136	0.292

R Squared = 0.938 (Adjusted R Squared = 0.932)

Table 3.13. Results of ANCOVA testing statistical significance of time and sucrose concentration in **mutans streptococci*** exposed to **20 or 100 mM sucrose**.

Source of Variation	SSIII	*df*	MS	F	*p*
Sucrose	108.681	2	54.340	33.085	**< 0.001**
Time	68.565	1	68.565	41.746	**< 0.001**
Sucrose x time	44.201	1	44.201	26.912	**< 0.001**

R Squared = 0.950 (Adjusted R Squared = 0.946)

Table 3.14. Results of ANCOVA testing statistical significance of time and sucrose concentration in **total *Lactobacillus* spp.*** exposed to **20 or 100 mM sucrose**.

Source of Variation	SSIII	*df*	MS	F	*p*
Sucrose	178.043	2	89.022	896.161	**< 0.001**
Time	1.180	1	1.180	11.882	**0.001**
Sucrose x time	1.841	1	1.841	18.535	**< 0.001**

R Squared = 0.997 (Adjusted R Squared = 0.997)

Type III sum of squares, SSIII; degrees of freedom, *df*; mean square, MS; F = (MS between groups)/(MS within groups). *$\log_{10}$ transformed prior to analysis. **bold** font denotes difference is statistically significant.

3.3.5 Plaque pH response to sucrose exposure

Biofilm pH initially rose from pH 6.72 to pH 6.96 within 10 seconds of the addition of saline while the addition of 20 mM and 100 mM sucrose elicited a Stephan-like pH curve (Figure 3.9). In other words pH initially dropped before rising again. Biofilm pH fell at a slow rate when exposed to saline so that the final pH at 2 minutes was pH 6.66. At 20 mM sucrose, pH was lowest (pH 5.85) 20 seconds after exposure and began to rise after 10 seconds; in other words from 30 seconds post exposure; before finally reaching pH 6.34 at 2 minutes. Only with the addition of 100 mM sucrose was critical pH (pH 5.5) reached approximately 50 seconds after exposure. By the eightieth second pH had reached 4.84 and remained low so that the final pH measured at 2 minutes was pH 4.82.

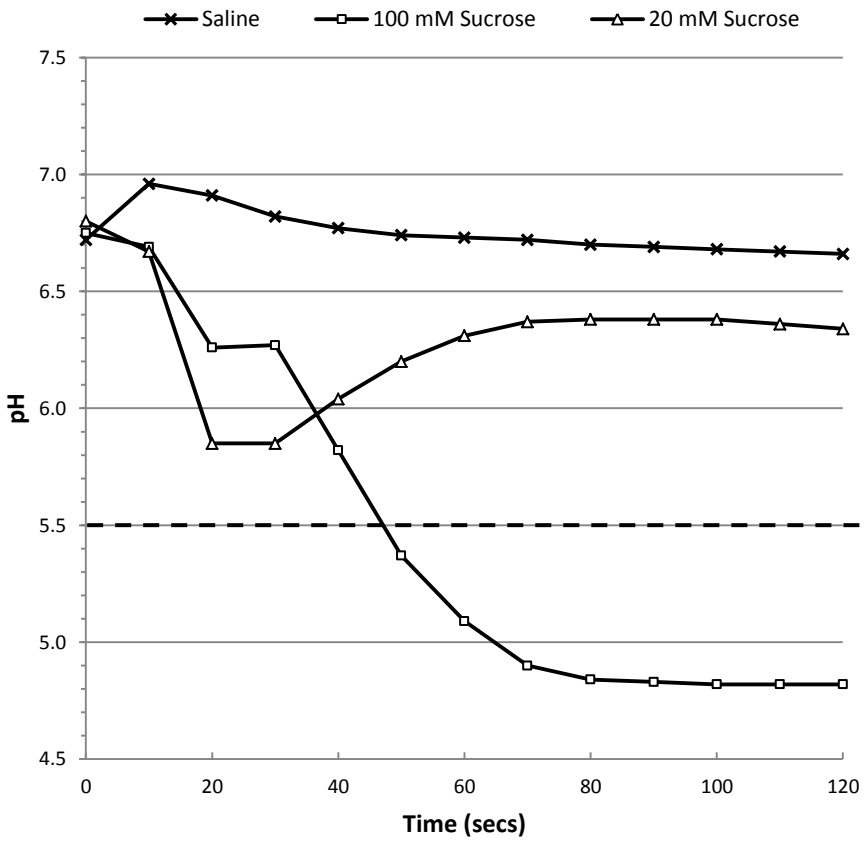


Figure 3.9. pH response of multispecies biofilm to the addition of saline, 100 mM sucrose or 20 mM sucrose. Dashed horizontal line indicates critical pH.

3.3.6 Relationship between QLF and TMR in quantifying caries

Both QLF and TMR detected similar degrees of change in the samples quantified accompanied by similar degrees of variation across replicates at each time point and in each group (Figure 3.5) therefore, the correlation between TMR ΔZ and QLF ΔF was examined. When comparing between the ΔZ and ΔF quantified for all individual replicate samples regardless of sucrose exposure $r = -0.8$, $p < 0.01$, $n = 37$ (Figure 3.10). Though the correlation coefficient is relatively strong and it was found that the regression model predicts the outcome variable significantly well ($p = 0.001$, $df = 1$, $F(1,36) = 52.107$), adjusted r^2 determined that only 58.7% of ΔF could be explained by ΔZ or vice-versa and the Durbin-Watson test revealed the presence of autocorrelation ($d = 1.199$, $n = 37$, $k = 2$). However, since evidence of a proportional relationship between the variables was provided, data was absolute $\log_{10}$ transformed prior to subsequent regression analysis so that assumptions of normal distribution, homoscedasticity and equal variance were met.

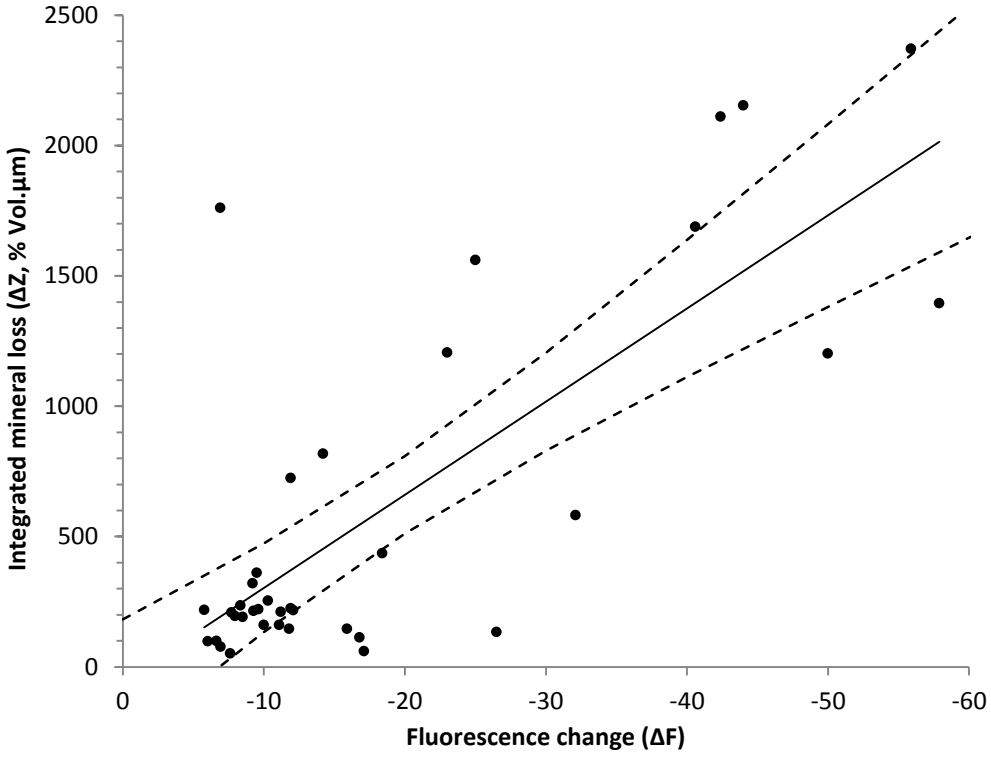


Figure 3.10. Scatter plot of the correlation between fluorescence change assessed by QLF and integrated mineral loss measured using TMR in a biologically relevant caries model of snacking consumption. Dashed lines represent upper and lower 95% confidence bounds.

Log transformed data revealed that r = -0.686, $p < 0.01$, n = 37 (Figure 3.11) and that a statistically significant directly proportional relationship exists (p = 0.001, df = 1, F (1,36) = 31.073) between variables. However, adjusted r^2 determined that only 45.5% of ΔF could be explained by ΔZ or vice-versa and the Durbin-Watson statistic again evidenced the presence of autocorrelation (d = 1.187, n =37, k = 2). Further inspection of the data set revealed that one cannot be confident that a directly proportional relationship exists between ΔF and ΔZ in 20 mM sucrose samples (Figure 3.12a) since the p of logarithmic data = 0.547 (n = 19, F (1,18) = 0.379) and the correlation is weak for this group (r = 0.148) while it was inconclusive that each observation is independent (d = 1.511, n = 19, k = 2). The relationship between ΔF and ΔZ in samples exposed to 100 mM sucrose (Figure 3.12b) was 0.676 and the regression model predicted the outcome variable significantly well (df = 1, p = 0.002, F (1,17) = 13.460) when tested as logarithms, however, adjusted r^2 was revealed as only 42.3% and d = 1.106, n = 18, k = 2 suggesting autocorrelation testing was inconclusive again.

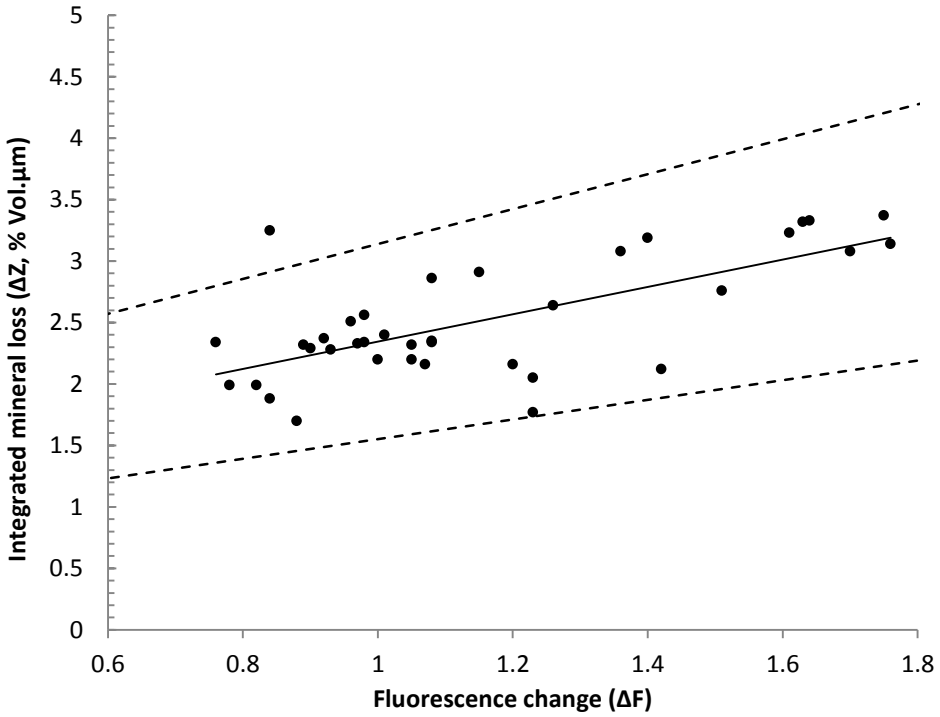


Figure 3.11. Scatter plot of the correlation between logarithmic fluorescence change assessed by QLF and logarithmic integrated mineral loss measured using TMR in a biologically relevant caries model of snacking consumption. Dashed lines represent upper and lower 95% confidence bounds.

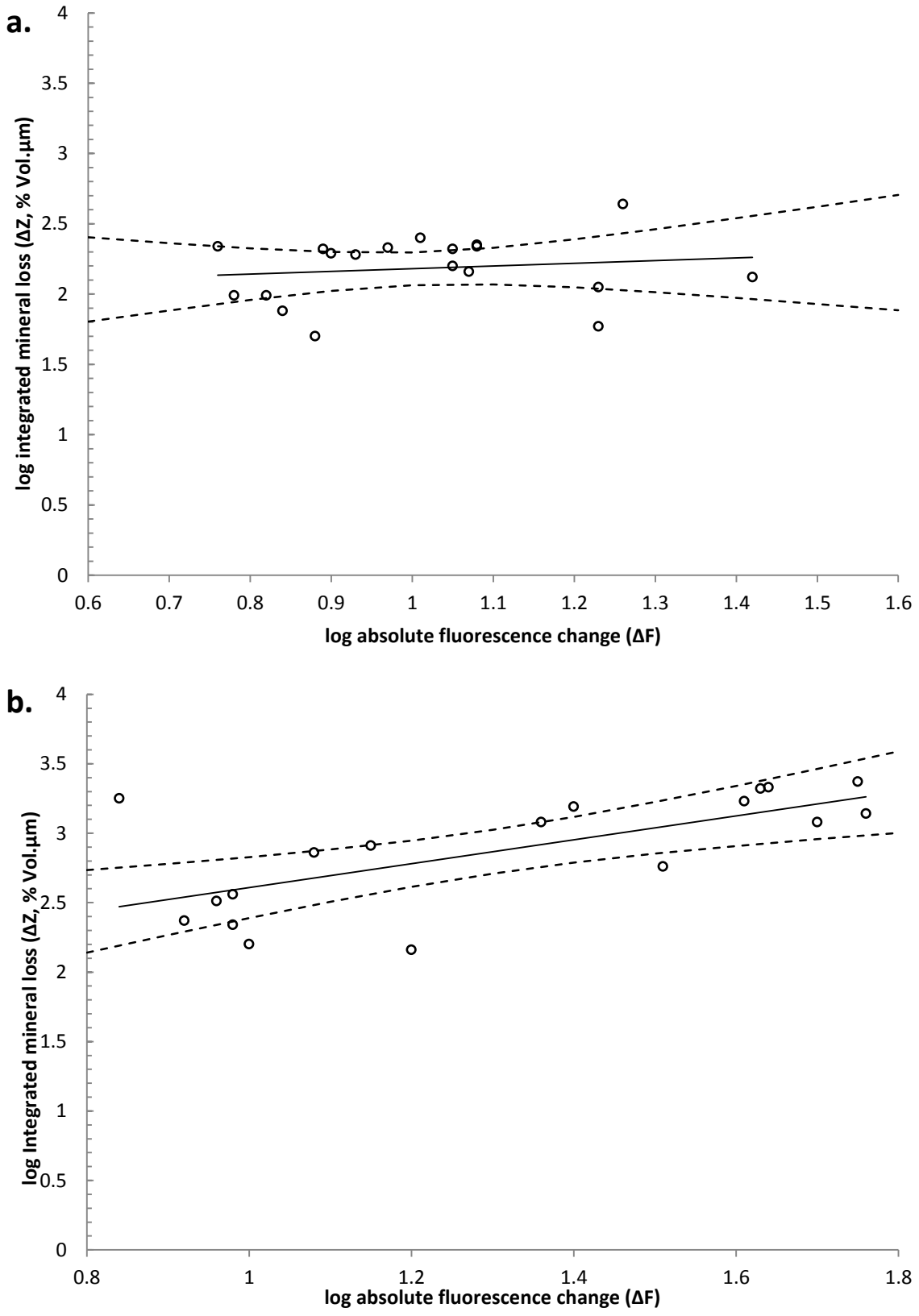


Figure 3.12. Scatter plot of the correlation between logarithmic fluorescence change assessed by QLF and logarithmic integrated mineral loss measured using TMR in a biologically relevant caries model of snacking consumption (a) 20 mM (b) 100 mM sucrose exposed. Dashed lines represent upper and lower 95% confidence bounds.

Finally, when comparing % change in average ΔF and average ΔZ over time relative to day 4 observations for 100 mM sucrose samples alone (Figure 3.13), r was 0.862 (n = 6) and it was shown that this regression model predicts the outcome variable significantly well (p = 0.027, df = 1, F (1,5) = 11.589) providing evidence of a straight line relationship exists between variables. The adjusted r^2 revealed that 67.9% of ΔF can be explained by ΔZ.

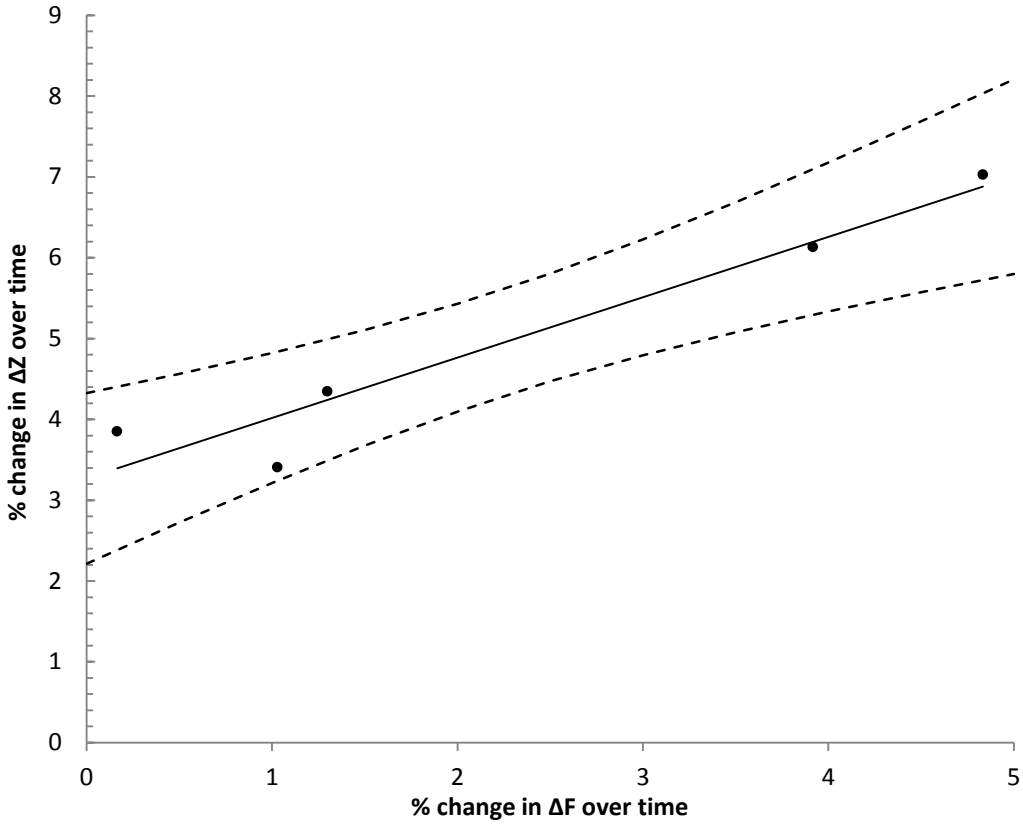


Figure 3.13. Scatter plot of the correlation between percent change in fluorescence over time and percent change in integrated mineral loss in a biologically relevant caries lesions exposed to multispecies biofilm pulsed 8 times daily with 100 mM sucrose. Dashed lines represent upper and lower 95% confidence bounds.

3.3.7 Replicate study investigating the effect of 100 mM sucrose on enamel demineralisation and the relationship between QLF & TMR in quantifying caries

The effect of 100 mM sucrose on enamel demineralisation was modelled in a repeat experiment in order to properly address the aim of investigating the concordance between QLF and TMR. This was required since it was suspected that correlations between these methods were influenced by problems that arose in preparing enamel sections for TMR in the previous study. Both QLF fluorescence change (ΔF, %) and mineral loss (ΔZ, %Vol.μm) measured by TMR analysis revealed a marked progression in caries over time in samples beneath biofilm pulsed with 100 mM sucrose 8 times daily (Figures 3.14 – 3.16 and Table 3.15). The largest change in ΔF (-48.701 ± 7.299, n = 2), in ΔZ (2517.50 ± 440.06, n = 2) and in lesion depth (86.98 ± 7.40 μm, n = 2) was observed in samples at day 14. It was subsequently revealed that changes over time were statistically significant for these outcome measures (Table 3.16), providing evidence that the development of caries was continuous throughout the experiment. NCSP of the enamel surfaces detected surface loss in all samples and the highest loss of 0.029 ± 0.079 μm SD (n = 3) was associated with samples at day 8. Differences in surface loss with and without 100 mM sucrose were statistically significant (p = 0.931, F(1,32) = 0.008) yet changes over time were not statistically significant and no statistical significance was derived when this dataset was compared to samples pulsed with no sucrose (see previous study; Section 3.3.3). p = 0.411, F(1,32) = 0.695 for time and p = 0.452, F(1,32) = 0.579 for the interaction of these factors; r^2 = 0.038). Multiple comparisons testing (Table 3.17) revealed that differences when comparing day 14 samples with those of both day 4 and day 6 were statistically significant for ΔF, ΔZ and lesion depth. When comparing day 12 with day 4 samples differences were statistically significant only in terms of ΔF and lesion depth. Additionally, differences between day 8 and 14 samples were statistically significant only for lesion depth whereas ΔF was the only parameter with statistically significant differences for day 4 vs 8 and 10 as well as day 6 vs 12.

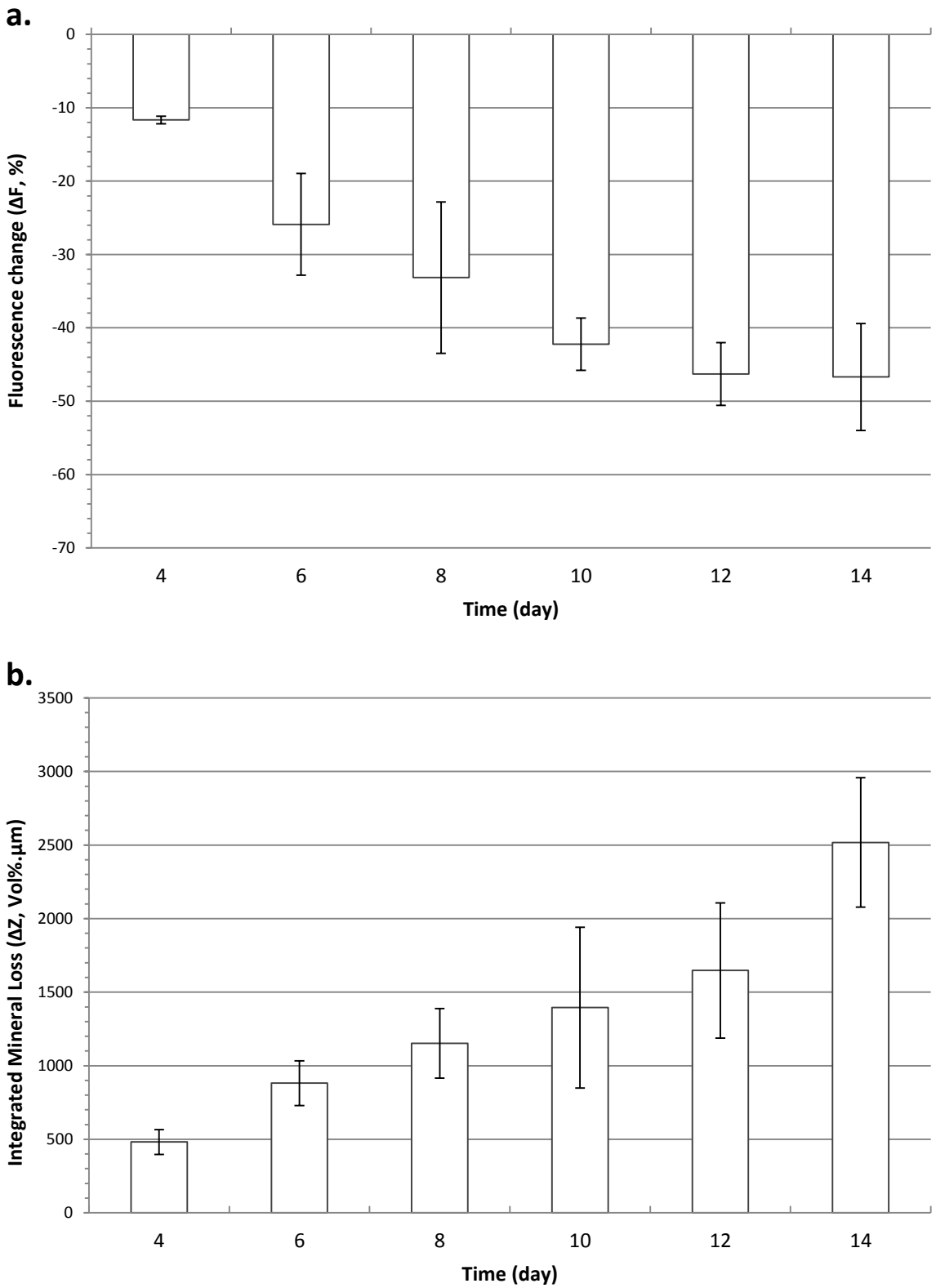


Figure 3.14. Longitudinal changes in (a) enamel fluorescence (average fluorescence loss, ΔF, %) as detected by QLF *in vitro* and (b) mineral loss (average ΔZ, %Vol.µm) determined by TMR in bovine enamel samples exposed to multispecies oral biofilm pulsed with 100 mM sucrose 8 times daily. Error bars represent standard deviation, $n = 3$ ΔF, $n = 2$, ΔZ. * means $p < 0.01$.

Table 3.15. Lesion depth (μm) determined by TMR in bovine enamel samples exposed to multispecies oral biofilm pulsed with 100 mM sucrose 8 times daily. Data is for mean ± standard deviation, $n = 2$

Sucrose conc.	Time (day)					
	4	6	8	10	12	14
100 mM	24.44 ± 6.82	36.63 ± 3.32	44.07 ± 6.81	54.82 ± 14.62	63.05 ± 10.07	86.98 ± 7.40

Figure 3.15. Typical QLF-D images of bovine enamel sampled longitudinally within a biological caries model subjected to 100 mM sucrose 8 times daily.

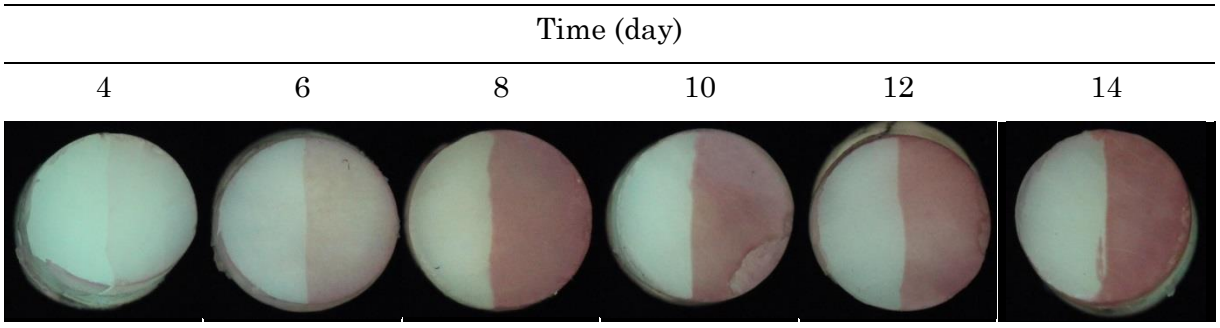
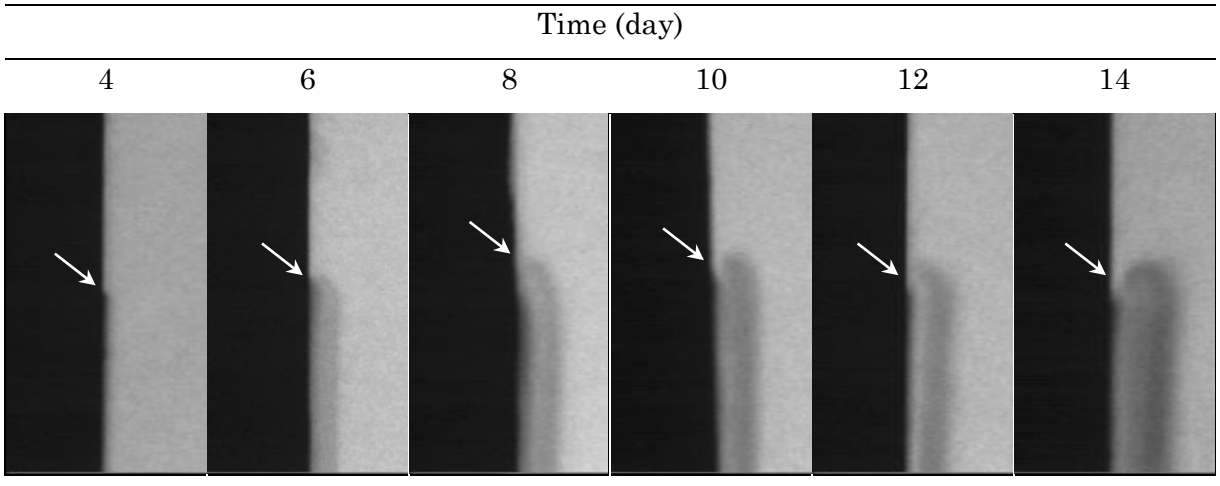


Figure 3.16. Typical TMR microradiography images of bovine enamel sampled longitudinally within a biological caries model subjected to 100 mM sucrose 8 times daily. Arrow indicates the interface between the biofilm exposed and nail-varnish protected zones.



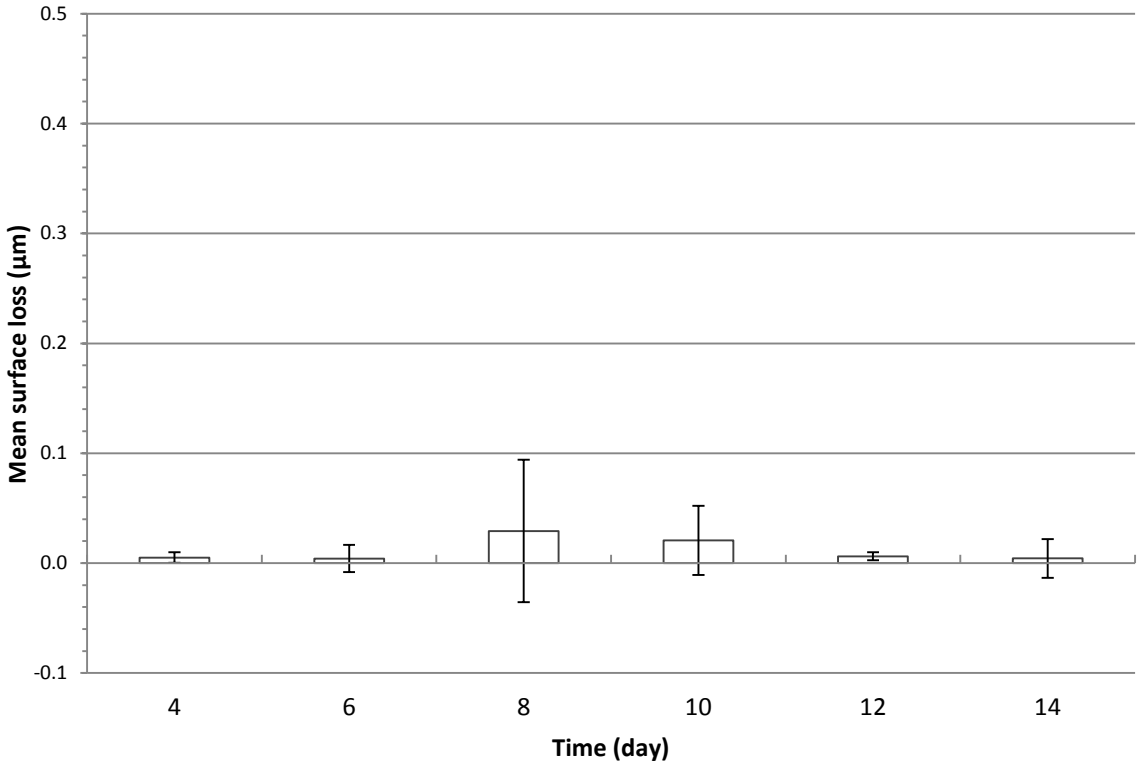


Figure 3.17. Mean surface loss (µm) of bovine enamel samples exposed to 100 mM sucrose 8 times daily as detected by NCSP measurements of height differences between negative control and treatment zones within samples. Error bars represent standard deviation, n = 3.

Table 3.16. Results of statistical significance testing the effect of time on changes in enamel caries characteristics over 14 days in a caries model exposed to **100 mM** sucrose 8 times daily according to fluorescence change (n = 3), mineral loss (n = 2), lesion depth (n = 2) and surface loss (n = 3).

Fluorescence change (ΔF, %)			Mineral Loss *(ΔZ, %Vol.µm)*			Lesion Depth (µm)			Surface Loss* (µm)		
F	R^2	p	F	R^2	p	F	R^2	p	F	R^2	p
(5, 12) 14.216	0.795	**< 0.001**	(5, 6) 6.612	0.718	**< 0.001**	(5, 6) 11.127	0.822	**< 0.001**	(5, 12) 0.348	0.237	0.874

F-statistic, F; proportion of total variance explained by the model, R^2

*natural $\log_{10}$ transformed prior to analysis; **bold** font emphasises statistical significance

Table 3.17. Results of multiple comparisons testing the statistical significance between changes in enamel caries characteristics over 14 days in a caries model exposed to **100 mM** sucrose 8 times daily according to fluorescence change (n = 3), mineral loss (n = 2), lesion depth (n = 2) and surface loss (n = 3).

Time (day)	Fluorescence change (ΔF, %)			Mineral Loss *(ΔZ, %Vol.μm)*			Lesion Depth (μm)			Surface Loss* (μm)		
	MD	±CI$^{95\%}$	p	MD	±CI$^{95\%}$	p	MD	±CI$^{95\%}$	p	MD	±CI$^{95\%}$	p
4 vs 6	14.2	-3.10, 31.5	0.133	-400.3	-1953, 1152	0.893	-12.2	-49.5, 25.1	0.777	.00045	-.0349, .0358	1.00
4 vs 8	21.5	4.17, 38.82	**0.013**	-670.9	-2224, 881	0.566	-19.6	-56.9, 17.7	0.385	-.0097	-.0451, .0256	0.932
4 vs 10	30.6	13.3, 47.9	**0.001**	-913.8	-2467, 639	0.304	-30.4	-67.7, 6.92	0.112	-.0066	-.0419, .0287	0.987
4 vs 12	34.6	17.3, 51.9	< **0.001**	-1240	-2793, 313	0.120	-39.9	-77.2, -2.64	**0.037**	-.0005	-.0358, .0348	1.00
4 vs 14	35.0	17.7, 52.4	< **0.001**	-2036	-3589, -483.5	**0.015**	-62.5	-99.8, 25.2	**0.004**	-.0004	-.0350, .0357	1.00
6 vs 8	7.27	-10.05, 24.6	0.721	-270.6	-1823, 1282	0.976	-7.43	-44.7, 29.9	0.959	-.0102	-.0455, .0251	0.919
6 vs 10	16.4	-0.97, 33.7	0.068	-513.4	-2066, 1039	0.770	-18.2	-55.5, 19.1	0.460	-.0070	-.0424, .0283	0.982
6 vs 12	20.4	3.08, 37.7	**0.018**	-839.9	-2393, 713	0.372	-27.8	-65.1, 9.54	0.153	-.0009	-.0363, .0344	1.00
6 vs 14	20.8	3.50, 38.14	**0.016**	-1636	-3189, -83.2	**0.040**	-50.3	-87.6, -13.0	**0.013**	-.0001	-.0354, .0353	1.00
8 vs 10	9.08	-8.24, 26.4	0.522	-243	-1796, 1310	0.985	-10.8	-48.1, 26.5	0.846	.0032	-.0322, .0385	1.00
8 vs 12	13.1	-4.19, 30.5	0.185	-569	-2122, 983	0.698	-20.3	-57.6, 17.0	0.366	.0093	-.0261, .0446	0.944
8 vs 14	13.5	-3.78, 30.9	0.164	-1365	-2918, 187	0.084	-42.9	-80.2, -5.61	**0.027**	.0101	-.0252, .0454	0.922
10 vs 12	4.05	-13.3, 21.4	0.965	-326	-1879, 1226	0.949	-9.56	-46.9, 27.7	0.895	.0061	-.0292, .0414	0.991
10 vs 14	4.46	-12.9, 21.8	0.948	-1122	-2675, 430	0.168	-32.2	-69.4, 5.15	0.091	.0070	-.0284, .0423	0.983
12 vs 14	.412	-16.9, 17.7	1.00	-796	-2349, 757	0.417	-22.6	-60.0, 14.7	0.282	.0009	-.0345, .0362	1.00

Mean difference, MD; lower and upper confidence intervals, ±CI$^{95\%}$

*natural $\log_{10}$ transformed prior to analysis; **bold** font denotes difference is statistically significant

In this repeat experiment both QLF and TMR detected similar degrees of change in the samples quantified accompanied by similar degrees of variation across replicates at each time point (Figure 3.14) therefore, the correlation between TMR ΔZ and QLF ΔF was examined once again. In this case, Pearson's product moment coefficient (r) was stronger than before r = -0.9, p = 0.001, n = 12) when comparing between the ΔZ and ΔF quantified for all individual replicate samples (Figure 3.18). Furthermore, the regression model predicts the outcome variable significantly well (p = 0.001, df = 1, F (1,11) = 30.120); providing evidence of a proportional relationship; while adjusted r^2 determined that 72.6% of ΔF could be explained by ΔZ or vice-versa.

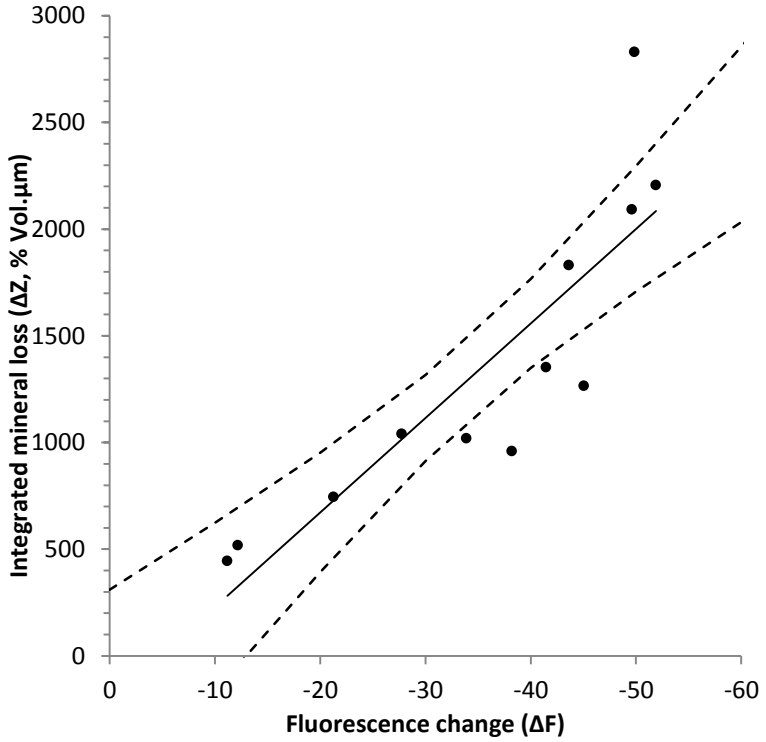


Figure 3.18. Scatter plot of the correlation between fluorescence changes assessed by QLF and integrated mineral loss measured using TMR in a biologically relevant caries model of snacking consumption. Dashed lines represent upper and lower 95% confidence bounds.

3.4 DISCUSSION

The caries model presented in this chapter was able to demonstrate dose response when multispecies biofilm on enamel were exposed to sucrose of different concentrations. The experiments of this study showed that degrees of enamel demineralisation were greatest in 100 mM sucrose exposed samples and that both 20 mM and 100 mM sucrose influenced the microbial dynamic of mutans streptococci and *Lactobacillus* spp. within multispecies biofilm compared to non-sucrose exposed biofilm.

The importance of the frequency of sugar consumption in caries has been established (Gustafsson *et al.*, 1954; Ccahuana-Vásquez *et al.*, 2007). No other studies have compared the longitudinal effects of 20 mM sucrose- and 100 mM sucrose-fed biofilm exposed 8 times daily on enamel caries *in vitro*. Despite this, a number of studies have demonstrated that the quantity of sucrose is also a relevant factor (Hefti and Schmid, 1979; Tehrani *et al.*, 1983; Aires *et al.*, 2006). There is an undeniable relationship between the two variables (Rugg-Gunn and Hackett, 1993). The *in situ* experimental procedure of Aires *et al.* (2006) comes closest to the concentrations tested in the present study and findings of each study are congruent. They were able to demonstrate that 5% (146 mM) or higher concentrations of sucrose have more cariogenic potential than 1% (29.2 mM) sucrose and that a threshold exists whereby concentrations of above 5% affect the acidogenicity and biochemical properties in the formation of biofilm capable of promoting dental caries development. 5% sucrose provided the same cariogenic potential as that observed for 10% (292 mM) and 20% (584 mM) sucrose.

3.4.1 Growth of bacterial members of multispecies biofilm in the presence of sucrose and its effects on enamel demineralisation

The growth of total bacteria, *Streptococcus* spp., mutans streptococci and *Lactobacillus* spp. all differed significantly whether multispecies biofilm were exposed to no sucrose or either 20 mM or 100 mM sucrose 8 times daily. Statistically significant differences according to time when comparing numbers of *Lactobacillus* spp. between all supplementation conditions reveal that the growth of this bacterial type is highly dynamic in response to sucrose and statistical significance in the interaction of sucrose and time suggest growth differs in markedly different manors at each time in each sucrose concentration condition. The same interpretation can be applied when comparing differences in mutans streptococci growth between biofilm in the absence of sucrose with those exposed to 100 mM and when comparing growth in 100 mM with that of 20 mM sucrose exposed biofilm. However, no statistical significance according to time as well as its interaction with sucrose in mutans streptococci growth in the absence of sucrose compared to 20 mM sucrose suggests bacteria of this group proliferated in similar ways in both circumstances.

20 mM sucrose enriched the growth of both mutans streptococci and *Lactobacillus* spp. compared to unpulsed control multispecies biofilm. Increases in proportions of *Lactobacillus* spp. may indicate an increase in biofilm acidification in the presence of sucrose of either concentration compared to when no additional sucrose was present. *Lactobacillus* spp. and mutans streptococci are highly aciduric bacteria often associated with caries due to their ability to metabolise and survive in acidic conditions compared to other oral bacteria. The proton-translocating ATPase developed by members of both bacterial groups, means they are capable of maintaining their intracellular pH between 0.5 and 1 units higher than the extracellular environment (Bowen, 2002). Pulsing with 100 mM sucrose resulted in lower microbial counts of mutans streptococci over time within multispecies biofilm

whilst slightly supplementing the growth of *Lactobacillus* spp. compared to unpulsed control multispecies biofilm. Regardless of the early limitation of mutans streptococci growth in 100 mM sucrose, by the eighth day bacterial counts of this group were similar to day 8 counts when biofilm was exposed to no sucrose, but were still lower than was recovered from 20 mM sucrose exposed biofilm. The bacteria recovered by this day in 100 mM sucrose are likely to be those that were most well adapted to the rapid transport and metabolism of sucrose and conditions of environmental acidification. It has been reported that *L. casei* is more resistant to environmental acidification, with gross membrane damage only evident at pH values lower than 3.0, whereas it appears *S. mutans*, is moderately aciduric in comparison, lacking barriers to small molecules at pH values significantly below 4.0 (Bender *et al.*, 1986). In this respect, the membrane ATPases constitute major workhorses of acid tolerance for oral streptococci in particular since *S. mutans* can operate glycolytic functions at pH as low as 4.0, functions which directly relate to their cariogenicity. Therefore these mechanisms inherently protect acid-sensitive glycolytic enzymes in the cytoplasm by moving protons out of the cell, through the ATPase components of the cell membrane. The final result is that glycolysis, and so cariogenicity, are more aciduric than functions involved in cell growth (Bender *et al.*, 1986). This selective advantage is pivotal in their survival within acidic environments, such as dental plaque post sucrose exposure, and this has been demonstrated in the present study in the growth trends of *Lactobacillus* spp. in both concentrations of sucrose and in mutans streptococci grown in 20 mM sucrose. Nonetheless, the minimum pH in dental plaque is not often lower than slightly below pH 4.0 (Schachtele and Jensen, 1982), but subtleties of the diverse microenvironments within dental plaque may be the source of persistence by bacteria that are well adapted to conditions of acidification.

Though it has been shown that mutans streptococci can also preferentially grow in the presence of acid and sucrose compared to other oral streptococci (Bowen, 2002), the lower bacterial counts observed under 100 mM sucrose pulsing conditions could be attributed to the ability of these organisms, *S. mutans* in particular, to produce EPS from sucrose. The production of sticky glucans is considered an essential expression of virulence and feature of cariogenicity in members of this bacterial group (Koo *et al.*, 2003; Koo *et al.*, 2005) and marked increases in its proportions would mean less physical volume is available for the bacterial cells. Nonetheless, its proven role in stabilising biofilm structure (Characklis and Cooksey, 1983), bacterial adhesion and accumulation at surfaces (Gibbons and Nygaard, 1968; Hamada and Slade, 1980; Freedman and Tanzer, 1982; Larrimore *et al.*, 1983; Gibbons, 1989; Rolla, 1989; Schilling and Bowen, 1992), gene transfer and intercellular communication as well as in the storage of extracellular carbohydrates that form a nutrient source when exogenous nutrients are limited (Wood, 1967; Gibbons, 1968; Manly and Richardson, 1968) mean the ability to synthesise EPS is an important selective advantage for specific bacteria while beneficially impacting other members of the biofilm consortia. In this regard the persistence and subsequent exponential growth of mutans streptococci; albeit at lower numbers when pulsed with 100 mM sucrose, can also be attributed to their active metabolism of EPS as an exogenous nutrient source. Though there is currently no known single enzyme capable of degrading all polysaccharides of the biofilm matrix (Flemming *et al.*, 2000), as biofilm becomes more complex with time, established food chains could facilitate the cooperative degradation of exopolysaccharides, ensuring the survival of a number of community members. Furthermore, the recent identification of a specific PTS expressed in biofilm of *S. mutans* fed with sucrose possibly involved in the transport and metabolism of carbohydrates within EPS, provides further impetus for *S. mutans* to preferentially produce EPS as a selective advantage (Ajdic and Chen, 2013). Perhaps most importantly, the production of more acids becomes the inherent consequence of

the bacterial degradation of these extracellular polysaccharides for use as a nutrient source. Since it seems EPS serves as an exogenous nutrient source under nutrient limitation it could be posited that between occasions of sucrose pulses the existing EPS formed during nutrient excess was broken down into acids ensuring prolonged acidification of the biofilm. In this respect, less EPS would have been formed by bacteria pulsed with 20 mM sucrose compared to 100 mM sucrose leading to less EPS available as nutrient source under nutrient limitation and less acid production overall. Elsewhere, it was shown that the frequency of sucrose exposure and its concentration appear to increase EPS concentration in biofilm matrices, lowering pH during fasting periods and enhancing demineralisation (Cury *et al.*, 1997; Ribeiro *et al.*, 2005; Aires *et al.*, 2006).

In this regard, the differential metabolism of sucrose and production of acids from a variety of sources by the biofilm bacterial community on a whole would lead to the distinctive difference in enamel demineralisation observed in the present study. Increased concentrations of these insoluble polysaccharides also lead to low concentrations of calcium, inorganic phosphorus and fluoride in plaque fluid (Cury *et al.*, 1997) meaning the biofilm environment would favour demineralisation. Indeed, the model presented here discriminates dose response on enamel demineralisation and caries initiation as determined by QLF-D, TMR and NCSP. 100 mM sucrose solution induced caries more quickly and formed more pronounced demineralisation curves than when biofilm was pulsed with 20 mM sucrose while no caries was detected in enamel beneath biofilm with no additional sucrose exposure. Both techniques revealed a statistically significant difference between indications of enamel demineralisation observed in 20 mM or 100 mM exposed samples at days 12 and 14 ($p < 0.0001$ for all). In samples exposed to 20 mM sucrose the difference in enamel demineralisation was statistically significant only at day 14 when compared

to non-sucrose pulsed samples as revealed by QLF ($p = 0.0004$). This finding reveals that caries was initiated only by day 14 when biofilm was exposed to 20 mM sucrose 8 times daily but supports that under this condition biofilm was slightly more cariogenic than if biofilm had no additional sucrose. The slow progression of caries is further demonstrated since differences in enamel over time, when exposed to 20 mM sucrose or no sucrose, were not statistically significant. Therefore, enamel from the beginning and the end of the experimental time course did not differ significantly under both conditions. On the other hand when exposed to 100 mM sucrose significant differences throughout the time course (Table 3.16) provides evidence that caries was initiated earlier and progressed more rapidly than in 20 mM sucrose and non-sucrose exposed samples.

In addition to reduced EPS formation, the lessened cariogenic impact of biofilm fed with 20 mM sucrose 8 times daily is also likely due to the activity of a number of members of this multispecies community capable of modulating overall plaque cariogenicity via diverse pathways. For example, the transformation of acidic molecules into less acidic end-products, as demonstrated in the consumption of non-volatile lactate to form propionate, acetate and carbon dioxide by *Veillonella* spp. (Mikx and van der Hoeven, 1975; Distler and Kröncke, 1980). As well as the aerobic degradation of lactate to acetate and carbon dioxide via pyruvate and lactate oxidase by isolates of *Neisseria* (Hoshino *et al.*, 1976). Since acetic and propionic acids are comparatively weaker than lactic acid produced by homofermenters in sugar metabolism, the consequence of this activity is biofilm with less cariogenic potential. On the other hand, biofilm neutrality may also be restored via the formation of amines or ammonia. Multispecies plaque is capable of decarboxylating amino acids such as arginine, aspartic and glutamic acids, histidine, lysine and ornithine, to form carbon dioxide and the respective amine (Hayes and Hyatt, 1974). *Fusobacterium*

nucleatum, *Prevotella melaninogenica*, *Treponema* species and peptostreptococci, among others, can ferment amino acids and release ammonia along with volatile end products such as butyrate and acetate (Newbrun, 1983). Despite sporadic research attention, the disposal of acids and the generation of alkali play an essential role in dental plaque physiology since many oral microorganisms are found in relatively large amounts in dental plaque despite having poor acid tolerance (Marquis *et al.*, 1987). Therefore, suggesting that these community members have developed methods to overcome acidic environmental pressures in order to survive. A highly effective mode of proton removal from plaque involves the incorporation of two protons from lactic acid into a proline ring to form delta amino valeric acid (Stickland, 1934; Curtis *et al.*, 1983). This amino acid was found to be abundant in dental plaque and the major microorganisms utilising this reaction appear to be peptostreptococci (Curtis and Eastoe, 1978). Many microorganisms lacking in acid tolerance mechanisms survive according to the arginine deiminase system; a pathway that involves the generation of ammonia and carbon dioxide to prevent the pH of the immediate environment from dropping too low (Marquis *et al.*, 1987; Curran *et al.*, 1995; Curran *et al.*, 1998). However, though this pathway is inducible by low pH, it is suppressed when glucose is present. Finally, a major source of alkali in the mouth is the degradation of urea which is constantly present in small amounts within saliva (Kleinberg, 1967) and many microorganisms including *S. salivarius* and actinomyces produce urease (Sissons *et al.*, 1988). In fact, urease activity in *S. salivarius* biofilms was enriched by as much as 130-fold in comparison to fluid chemostat cultures grown in similar conditions (Li *et al.*, 2000). An inverse relationship was demonstrated between the ureolytic capacity of dental plaque and cariogenicity (Clancy *et al.*, 2000).

The differential pH responses of biofilm to sucrose concentration observed in the current study support this theory and agree with Imfeld (1983); who showed that

there is a relationship between sucrose concentration and pH decreases in dental plaque *in vivo*. The moderated fall in pH in biofilm exposed to 20 mM sucrose provides evidence for a number of aspects of microbial metabolism potentially occurring in response to this concentration of carbohydrate. It is likely a consequence of a combination of; the activity of members of the multispecies biofilm capable of acid disposal and alkali generation, the reduced production of acidic products as well as the proportions of weaker (high pKa, e.g. propionic and acetic) and stronger (low pKa, e.g. lactic) acids produced. This and the result that values below critical pH were observed only in biofilm exposed to 100 mM sucrose provides further direct explanation of the enamel demineralisation pattern observed in this study. It would appear that in the presence of 100 mM sucrose community members of the biofilm produce higher amounts and proportions of strong acids. It has been shown that artificial caries lesions were elicited from both lactic and acetic acids and that combinations of the two caused additive demineralisation effects (Featherstone and Rodgers, 1981). Furthermore, it has been suggested that the diffusion of acetic acid into enamel may be facilitated by the production of lactic acid (Featherstone *et al.*, 1979). Here it was claimed that acetic acid is protonated, diffusing into enamel and dissociating again to form a subsurface lesion, once plaque pH is lowered by lactic acid. This is supported in further studies (Edgar, 1983; Geddes *et al.*, 1984) wherein the preferential uptake of acetic acid by enamel was demonstrated and falls in acetate concentrations during pH falls (Dodds and Edgar, 1988). Likewise, volatile acids could be responsible for buffering in dental plaque, modifying the fall in pH caused by low pKa acids (Vratsanos and Mandel, 1982). Considering that plaque pH is generally higher than would be expected when concentrations of lactic acid are known, a computer model calculated pH values more typical of plaque pH once acetic acid concentrations were also taken into account (Margolis *et al.*, 1985). Despite factors such as salivary buffering and plaque base production, correlations between plaque lactate and acetate concentrations with pH response provide support for the

statement that the utmost important factor affecting dental plaque pH is carbohydrate from food (Dodds and Edgar, 1988).

Once again, the proportions of *Lactobacillus* spp. provide an indication of the possible increased acidification of the biofilm when exposed to 100 mM sucrose compared with when developed in the absence of additional sucrose. However, average quantities over time of *Lactobacillus* spp. were higher in 20 mM sucrose biofilm compared to biofilm pulsed with 100 mM sucrose. Since these experiments were undertaken on separate occasions, therefore involving the propagation of biofilm from separate aliquots of saliva pool, it is difficult to directly compare results. In the past a degree of variability has been reported both in a defined biofilm model (Kinniment *et al.*, 1996b) and in microcosm based studies (Pratten *et al.*, 1998b) using CDFF technology. Hence, this model has the potential for further development to reduce variability and streamline the operation of the CDFF in biofilm investigations. Nonetheless, the observed mineral loss of the associated enamel samples provides clarity regarding the cariogenic potential of each biofilm. The discrepancy between the viable proportions of known cariogenic bacteria and the enamel demineralisation trends supports the argument that the behaviour of biofilm community members in response to environmental stresses is the most important factor in ascertaining their specific role in the cariogenicity of biofilm. The quantity of specific bacterial species in a given oral biofilm cannot be a primary indicator of caries risk. This is supported in studies of a number of cariogenic features of dental plaque bacteria such as in acid formation, sugar metabolism and in EPS formation. An example of the latter is in studies revealing that the demineralisation of enamel was greatest when artificial biofilm analogous to dental plaque consisted of 95% EPS and only 5% *S.mutans* compared to other ratios with higher proportions of bacteria (Zero *et al.*, 1986a). In this case, the findings of the studies of this chapter support earlier claims that the outright

association of *S. mutans* to caries activity needs to be re-evaluated (Beighton, 2005). Furthermore the experimental outcomes support the ecological plaque hypothesis; the currently accepted perspective in caries aetiology (Marsh, 1994, 2003a).

Nanometre scale surface changes were observed in dental enamel beneath biofilm fed with sucrose 8 times daily and differences were statistically significant according to sucrose concentration. Trends were in line with those observed by the QLF and TMR indications of enamel demineralisation with less surface loss observed in samples exposed to 20 mM sucrose than to 100 mM sucrose. Additionally, as in QLF and TMR observations of greatest enamel demineralisation, greatest surface loss was in day 12 samples exposed to 100 mM sucrose. Despite proven use in the non-destructive study of enamel erosion (Zhang *et al.*, 2000; Hemingway *et al.*, 2010; Theocharopoulos *et al.*, 2010), the application of NCSP in the study of dental caries in tooth enamel has been limited. This is likely because caries, unlike erosion, develops in subsurface regions of dental tissue. Nonetheless, as caries progresses it has been noted that surface changes occur, namely increases in enamel porosity (Haikel *et al.*, 1983; Holmen *et al.*, 1985; Holmen *et al.*, 1987; Thylstrup *et al.*, 1994). A significant increase in the depth of pits and pores of enamel surfaces was demonstrated in a 3-month *in vivo* profilometric study, attributed to sites of retention of acids or chelating agents (Whitehead *et al.*, 1997). As in the current study, these authors report non-uniformity and prescribe that the configuration of the enamel surface could play an important role in mediating the development of lesion types. The prism-like structures resolved by NCSP in the present study, therefore mean the application of this technique in resolving subtle surface changes is promising and worth further testing. These structures can be attributed to the preferential dissolution of the crystalline phase by acidic products of the biofilm bacterial metabolism causing the protein-rich inter-rod space to become more prominent. This has been elucidated

elsewhere in citric acid etched enamel (Habelitz *et al.*, 2001) using atomic force microscopy and extensively using SEM after exposure to abiotic model systems (Habelitz *et al.*, 2001) and after the accumulation of dental plaque on enamel *in vivo* (Holmen *et al.*, 1985; Holmen *et al.*, 1987). Furthermore, in the model presented and in the natural setting it is possible that both erosion and caries occur simultaneously considering the increasing complexity of the modern diet. In this regard, this technique holds value in the design of this study and others like it as a way of confirming suspected erosion or erosion revealed from TMR investigations.

3.4.2 Relationship between QLF and TMR in caries quantification

It appears that both QLF and TMR detected similar degrees of change accompanied by similar degrees of variation in enamel and $r = 0.78$ when the correlation between TMR ΔZ and QLF ΔF was examined. However the relatively low r^2 from linear regression analysis revealed that based on this dataset only 58.7% of ΔF could be explained by ΔZ or vice-versa. This can be attributable to problems that arose in preparing the enamel sections for TMR. Though this technique is the long-standing gold-standard for the assessment of enamel mineralisation it involves extensive procedures so that planar-parallel approximately 80 μm thin sections of enamel are obtained. In the present study a number of sections appear thinner and slightly wedge-shaped, an unfortunate consequence of wear of some parts of the grinding apparatus. The TMR software is able to account for some deviations from the ideal but values would be inherently affected by the variation caused at the grinding stage. The statistically significant findings for both ΔZ and lesion depth at day 6 (100 mM) compared to day 4, 8 and 10 samples but not in terms of ΔF are an example of samples affected by the likely artefact of preparation bias. Furthermore, it would appear that QLF and TMR did not agree well when quantifying caries in 20 mM sucrose exposed enamel ($\log_{10}$ transformed; $r = 0.148$, $p = 0.5$) whilst correlation for

100 mM alone was stronger (r = 0.686) yet r^2 was low, 45.5%. This is likely due to the lack of caries lesions in 20 mM exposed samples in combination with variations introduced during the preparation of sections for TMR. Only when 100 mM samples alone were expressed as % change over time was r^2 relatively high (67.9%), suggesting this transformation is the best fit for the regression model to the dataset, and r in this case was strong, 0.862. Nonetheless, in a replicate of the 100 mM sucrose exposed study, where TMR equipment issues had been resolved, a stronger correlation was indeed observed between ΔF and ΔZ (r = 0.866, p = 0.001, n = 12). Furthermore this dataset provided evidence that a statistically significant proportional relationship exists between each variable and that a higher proportion of ΔF could be explained by ΔZ or vice-versa (72.6% vs 58.7% in previous experiment). These findings provide evidence that correlations of QLF with TMR can be significantly affected by issues that may arise due to the lengthy time-consuming preparation of enamel for analysis by the latter technique.

The observed Pearson's product moment coefficient of the present study (r = 0.866) indicates a fairly strong relationship between these methods of mineralisation analyses (relationship defined as strong when r is above 0.9). The heterogeneous nature of caries lesions formed by the complex multispecies biofilm and the difficulties in fully matching the areas assessed by both techniques could account for some of the disassociation quantified. Natural carious lesions can be highly variable in lesion depth and mineral loss and the slicing and subsequent grinding of enamel sections for TMR leads to some loss of lesion tissue. Since TMR assesses single or multiple slices to provide a representation of the lesion on a whole whilst QLF measures the whole lesion non-destructively, comparison of the two methods is problematic. In this way r values obtained may inherently be modulated by the TMR process. The work of the present study, therefore, provides evidence for this theory. Table 3.18 summarises

previously reported correlation coefficients to describe the relationship between QLF ΔF and TMR ΔZ of caries lesions derived from a range of demineralisation approaches in smooth surface caries models. Both laser induced fluorescence and light induced fluorescence studies were included since the two show strong correlation (r = 0.93) in the paired quantification of fluorescence loss (Al-Khateeb *et al.*, 1997b; ten Bosch,

Table 3.18. Summary of previously reported correlation coefficients between QLF ΔF and TMR ΔZ values for the quantification of a range of caries lesions.

Reference	Caries modelling technique	Enamel source	r	Optical source
Hafstrom-Bjorkman *et al.* (1992)	de-pH cycling	Human	0.86 (10)	Laser
Emami *et al.* (1996)	de-natural incipient lesions	Human	0.73 (36)	Laser
Al-Khateeb *et al.* (1997b)	de- re- pH cycling	Human & bovine	**0.79** (25/30)	Laser
			0.84 (25/30)	Light
Al-Khateeb *et al.* (1997a)	de-pH cycling re-*in situ*	Human	**0.76** (72)	Laser
Ando *et al.* (1997)	de-buffered soln.	Bovine	**0.69** (144)	Light
Hall *et al.* (1997a)	de-buffered soln.	Bovine	**0.83** (84)	Laser
Lagerweij *et al.* (1999)	de-buffered soln.	Human	**0.70** (40)	Laser
			0.63 (40)	Light
Ando *et al.* (1999)	de-buffered soln.	Human	**0.83** (77)	Laser
			0.86 (77)	Light
Ando *et al.* (2001)	de-buffered soln.	Human (permanent)	**0.53** (30)	Laser
			0.62 (30)	Light
Ando *et al.* (2001)	de-buffered soln.	Human (deciduous)	**0.84** (30)	Laser
			0.88 (30)	Light
Shi *et al.* (2001)	natural lesions	Human (premolars)	**0.69** (71)	Laser
Gmur *et al.* (2006)	defined biofilm batch culture	Bovine	0.82 (10)	Light
Bakht *et al.* (2014)	multispecies biofilm	Bovine	0.8 (37) 0.9 (12)	Light

Bias towards demineralisation and remineralisation in model = de- or re- respectively

r values in bold represent studies using TMR as gold standard technique for validation, non-bold are for studies comparing QLF with LMR.

Value in parentheses = *n,* sample size

2000) as well as those that employ the longitudinal variant of TMR, LMR. The correlation defined in the present study is similar to those reported when assessing artificial carious lesions in bovine enamel (r = 0.83) (Hall *et al.*, 1997a) or human enamel (r = 0.84) (Al-Khateeb *et al.*, 1997b); in a six-species defined biofilm caries model, (r = 0.82) (Gmur *et al.*, 2006); and in studies of early caries lesion formation in deciduous teeth; r = 0.88 respectively (Ando *et al.*, 2001). Though Hafstrom-Bjorkman *et al.* (1992) reported overall r as 0.86, a higher correlation coefficient of 0.97 was observed when comparing values for individual slices and not for averages that represent the entire lesion.

Nevertheless, it is important to note that correlations claiming to prove agreement are inherently flawed since this statistic describes the strength of relationship between two variables. It is difficult to apply statistical analyses designed for method validation (such as Bland-Altman/Tukey's mean difference plots) in this instance since both QLF and TMR do not produce values in the same units. In this sense a strong correlation can be an indicator of validation but claims cannot be made that it assesses agreement. Therefore, further efforts must be made to develop statistical models and improve current study design to prove validation more thoroughly.

3.4.4 CDFF inoculation

The work of this thesis focuses on the use of multispecies microcosm inocula in order to model a caries process that can be deemed somewhat more biologically relevant than *in vitro* non-biological caries models and existing *in vitro* biological models of caries. The model system chosen for these studies, the CDFF, has traditionally been inoculated by three methods; single species (Pratten *et al.*, 1998b), defined multispecies (Kinniment *et al.*, 1996a, b) and microcosm whereby a plaque sample or saliva pool is employed (Hope and Wilson, 2003; Pratten *et al.*, 2003; Hope and

Wilson, 2006). Experiments involving single species inocula have allowed researchers to study biofilms of a particular organism of interest but do not fully reflect the effect of interactions from other members of the microbial community. Therefore, this type of model is unrealistic of *in situ* oral biofilm. Defined multispecies cultures address this issue by allowing the study of specific interactions between chosen members of the oral biofilm and so far pseudo oral microbiota ranging from two (Dalwai *et al.*, 2006) to ten (Bradshaw *et al.*, 1996b) individual microbial members have been achieved. This type of inoculate can replicate the complex interactions that are known to occur in the oral ecosystem; though to a limited degree. Despite this, the defined nature of such an approach has its merits and has allowed the detailed study of microbial interactions within biofilm, proving a useful tool for modelling the oral microbial ecosystem (Bradshaw *et al.*, 1989; Kinniment *et al.*, 1996b). Collecting saliva or plaque samples from a number of individuals and combining them together to form a "saliva pool" from which aliquots can be derived circumvents variation that could occur by sampling from one donor. This pool serves to represent "average saliva" and ensures a greater degree of conformity between aliquots than if inoculates were derived from one donor since inherent variation exists even when sampling from one donor at different times. The microbial changes that occur when inoculating a CDFF with a microcosm community of oral bacteria has, to date, not been investigated. Therefore, the microbial dynamics of CDFF inoculation were investigated and results lead to further improvements in the way CDFF experiments are operated in the studies of this thesis. Typically, the CDFF has been inoculated over a 24 hr period or until all of the inoculated artificial saliva has been delivered into the CDFF system. However, results of this study reveal that all required organisms and specifically those associated with caries such as mutans streptococci and *Lactobacillus* spp. were sufficiently delivered by the twelfth hour. In other words inoculation is complete by the end of a working day. Additionally, results revealed that the conditions of this method of inoculation do not support the growth of sulfur

producers and black pigmented organisms beyond, at the latest, the eighth and third hours respectively. The intermittent zero cfu's observed in both populations in the first replicate experiment are likely due to their failure to grow on their respective culture plates and less so that there were none present at these times. Finally, although the growth curves of each bacterial population were similar in both experiments, the present study has illuminated subtle differences in quantities and growth rates of all bacterial populations investigated. Therefore, this work contributes to our understanding of microcosm CDFF inoculation and provides further insight into the possible implications of inoculating with separate aliquots of microcosm pooled saliva on biofilm development.

3.4.5 The use of hydroxyapatite and enamel substrata

High quality enamel tissue can be scarce (Mellberg, 1992) and the fragility of the tissue means fabricating sections can be difficult without causing defects in the enamel (Lagerweij *et al.*, 1996). Consequently, enamel samples that adhere to a planar-parallel discs specification are inherently expensive and their production is time costly therefore, hydroxyapatite discs have been used in their place as substrata for microbial growth (Bradshaw *et al.*, 1996b; Pratten *et al.*, 1998c; Hope and Wilson, 2003; Valappil *et al.*, 2014). It has also become widely accepted that hydroxyapatite is analogous to dental enamel (Driessens, 1982) due to similarities in their physical and chemical composition. Hydroxyapatite possesses electronegativity similar to that of enamel meaning the natural processes of pellicle formation and bacterial colonization on this substratum acceptably simulate those very processes on dental enamel. Only minor differences in single species biofilms of *S. sanguinis* were reported when developed on hydroxyapatite, enamel or PTFE substrata (Pratten *et al.*, 1998c) whilst no statistically significant differences between bacterial growth on hydroxyapatite or enamel in a model of multispecies biofilm was demonstrated (Owens, 2013). Despite

this, growth on hydroxyapatite results in differential gene expression when compared with growth on polystyrene, another negatively charged substratum (Shemesh *et al.*, 2010) but due to the chemical similarities between hydroxyapatite and enamel, it is anticipated that this effect would be reduced. Additionally, no significant differences with respect to viable bacterial counts and lactic acid production were reported when biofilm was exposed to no antimicrobials (Deng *et al.*, 2004). However, the biofilm pH and its response to antimicrobials was affected when comparing bovine dentine and poly-acrylate substrata (Deng *et al.*, 2004). These results suggest substrata may influence the sensitivity of microbial populations but not their ability to colonise these substrata. It has been theorised that buffering by minerals could potentially contribute to the acclimation of the microbial community and influence susceptibility (Zaura *et al.*, 2002; Deng *et al.*, 2004). Transformation competence with regards to calcium has been demonstrated in *S. pneumoniae* (Trombe *et al.*, 1992) so its release during demineralisation could play a role in gene transfer (Molin and Tolker-Nielsen, 2003).

3.5 CONCLUSIONS

The model described in the present study facilitated the holistic investigation of both multispecies biofilm and enamel caries. Findings support the ecological plaque hypothesis for caries aetiology since it would appear that caries resulted from a shift in the behaviour of biofilm bacteria in response to sucrose exposure and was not directly associated with the quantities of particularly cariogenic bacteria.

In summary, a dose-dependent response to sucrose was demonstrated in terms of both enamel demineralisation and the microbial ecology of multispecies biofilm. 100 mM sucrose solution induced caries in enamel more quickly and formed more pronounced demineralisation curves than in samples beneath biofilm pulsed with 20 mM sucrose,

as demonstrated by QLF and TMR and confirmed by NCSP. Although the growth of members of the caries associated bacterial groups; mutans streptococci were not limited in 20 mM sucrose and *Lactobacillus* spp. were enriched, pH remained stable post-sugar exposure and values were not below pH 5.5 (critical pH) in response. This can be attributed to the acid disposal and alkali generation capabilities of some members of the microbial community for survival as well as the likely reduced formation of EPS under these conditions. Biofilm pH values followed typical Stephan-curve formation and critical pH was reached quickly and remained low in response to 100 mM sucrose. However, quantities of mutans streptococci in biofilm exposed to 100 mM were lower than in non-sucrose pulsed biofilm in early days of the experiment whilst the growth of *Lactobacillus* spp. appeared to be supplemented. The enrichment of the latter group of bacteria served as further indication of increased biofilm acidification since *Lactobacillus* spp. are highly aciduric. Whereas, it was revealed that by the eighth day numbers of mutans streptococci were similar to those by the same day in non-sucrose exposed biofilm though still lower than those recovered from 20 mM sucrose exposed biofilm. Regardless of these differences, bacteria recovered by this day in 100 mM sucrose are likely those that were most well adapted to the rapid transport and metabolism of sucrose and conditions of environmental acidification. Therefore, the present study provides evidence of ecological adaptation. Early limitations in the quantities of mutans streptococci are attributable to the heightened formation of EPS under conditions of higher sucrose availability and the degradation of these insoluble polysaccharides between each sucrose pulse would lead to further and prolonged periods of biofilm acidification. Increased EPS formation has been directly associated with increased enamel demineralisation in previous studies. Therefore, the quantity of specific bacterial species in a given oral biofilm cannot be a primary indicator of caries risk. The behaviour of biofilm community members in response to environmental stresses is the most important factor in ascertaining their specific role in the cariogenicity of biofilm.

NCSP quantified nanometre scale surface loss from enamel beneath biofilm exposed to sucrose with statistically significant differences according to concentration at day 12. Highest ΔF, ΔZ and lesion depth were reported in samples of this day. This technique was also able to distinguish prism-like structures at the enamel surface consistent with those found by SEM and AFM in other demineralisation studies and attributable to the preferential dissolution of the crystalline phase by acidic products of the biofilm bacterial metabolism causing the protein-rich inter-rod space to become more prominent. Therefore, the application of this technique in resolving subtle surface changes, is promising and worth further testing.

The strong, statistically significant relationship observed between results from TMR and QLF for the quantification of enamel caries indicates that QLF is suitable for the *in vitro* measurement of mineral density changes in experimental enamel specimens. However, this is only the case when the preparation of enamel sections for analysis by TMR was optimal. Furthermore, a strong correlation can be an indicator of validation but claims cannot be made that it assesses agreement. Therefore, further efforts must be made to develop statistical models and improve current study design to prove validation more thoroughly.

Finally, elucidation of the microbial and pH dynamics of CDFF inoculation provides the conclusion that a 24 hour inoculation period is not essential when operating the CDFF. Results indicate that inoculation is fulfilled within 12 hours and justifies the use of this shorter inoculation period for subsequent studies. It was suspected that there is a level of variability in the biofilm derived from separate aliquots of saliva pool which leads to the conclusion that it would be worthwhile to further develop the CDFF model for use in comparative studies of biofilm and caries.

Chapter 4 A novel dual CDFF model

4.1 INTRODUCTION

In the past a degree of variability has been reported both in a defined biofilm model (Kinniment *et al.*, 1996b) and in microcosm based studies (Pratten *et al.*, 1998b) using CDFF technology. Containing many hundreds of microorganisms, microcosm inoculum provides the basis of the most representative modelling strategies; yet intrinsic variation is observed between microcosm samples derived from salivary and plaque inoculum even when obtained from a single individual. This variability limits the power of the model to elucidate small differences when comparing the effects of test agents; not to mention it has potential in undermining the reproducibility of some findings. Traditionally, combining saliva or plaque samples from a number of donors to form a 'pool' of organisms before dividing into aliquots that are essentially identical in composition viably attempts to reduce variation. Pooling leads to more conformity between aliquots of inoculate, and attempts to make certain that a wide range of bacteria are present in biofilms derived from it. It is logical to assume that this pool contains a highly diverse community of microorganisms, more so than if it were derived from a single individual, but it is often deemed representative of average saliva. Nonetheless, the tendency of bacteria to aggregate in clusters means these pools are not always homogenous and division into aliquots is not guaranteed to be consistent. Subsequently, minor variations in the bacterial composition of inocula leads to differences in the resulting microcosm community (Ledder *et al.*, 2006) and could be exaggerated by the conditions of the inoculation media, in turn, affecting biofilm formation. Extensive processing of the pool to avoid this is inappropriate since fragile microorganisms such as *Actinomyces* spp. (Hope and Wilson, 2003) can be damaged and strict anaerobes like *Prevotella* spp (Bradshaw *et al.*, 1996a) can undergo oxidative stress if exposed to high levels of fluid shear forces. Therefore, the

model requires further improvement and other solutions must be developed to address this issue of variability in CDFF operation. A "split CDFF" design appears to reduce these minor variations to improve reproducibility within studies comparing the effects of caries-preventative agents (Hoogenkamp *et al.*, 2003; Deng *et al.*, 2005). Here a standard fifteen pan CDFF was modified so it was inoculated normally during early biofilm growth then operated so that the turntable oscillated through a fixed arc of 180° as two different adjunct agents were supplied via separate inlets to each side of the CDFF (i.e. half the biofilm samples). This was achieved by the prior removal of four sample pans at opposite sides of the turntable, which was switched to a motor power supply with a reversing circuit to initiate this stage of the experiment. Though it was shown that there was no carry-over of bacteria or agents between the two sides, only eleven replicate biofilms are produced in this study design; five on one side and six on the other. It would be more suitable to operate two CDFFs concurrently fed by the same inocula while exposed to identical conditions; and if reduced variability is demonstrated, this model would be ideal for comparatively studying biofilm exposed to different treatments, such as controls compared to test agents.

Aims

The aim of this study was to develop a novel, dual CDFF protocol wherein experiments with replicate biofilm in two CDFFs were developed under the same incubation conditions and fed with the same inoculum and liquid medium supply. The aim was to investigate the microbiological variability between CDFFs run either in parallel or separately and to explore the use of such a model in elucidating the effects of sucrose on biofilm growth.

4.2 EXPERIMENTAL PROCEDURE

Two 8-pan CDFFs were fully loaded with forty, 5 mm hydroxyapatite (HA) discs (Clarkson Chromatography Products, South Williamsport, PA, USA) recessed to a depth of 200 μm and sterilised by autoclave as described in Section 2.4 and 2.5. The circuit diagram of the dual CDFF set-up is shown in Figure 4.1. An inoculation flask was prepared with one litre of artificial saliva growth medium as described in Section 2.6. Once this vessel had cooled to incubator temperature, an aliquot of the saliva pool (Section 2.2) was thawed and added directly into the inoculation flask whilst its contents were magnetically stirred. To ensure that the same inoculum entered both CDFFs at the same flow rate, a multichannel peristaltic pump (323 Du pump with 318MC head, Watson-Marlow Ltd., Falmouth, Cornwall, UK) was used to deliver liquid at 0.72 ml min^{-1} into each CDFF (Lamb *et al.*, 1991; Guyton *et al.*, 1997). Inoculum flow ceased after 12 hours at which point the flow of sterile artificial saliva medium was initiated at a flow rate of 0.38 ml min^{-1} per CDFF. Two CDFF experiments were carried out concurrently and two were carried out in series.

Sample pans were removed from both CDFFs simultaneously on days 4, 6, 8, 12 and 14. All five biofilm laden HA discs within each sample pan were extracted using sterile forceps taking care not to disrupt the biofilm. All 5 biofilm samples were vortex mixed for 30 seconds in 900 μl of PBS to dislodge the biofilm from the disc and serially diluted up to 10^6 using sterile PBS solution before being cultured for total viable bacteria, *Streptococcus* spp., *Lactobacillus* spp., mutans streptococci (Section 2.7.1.).

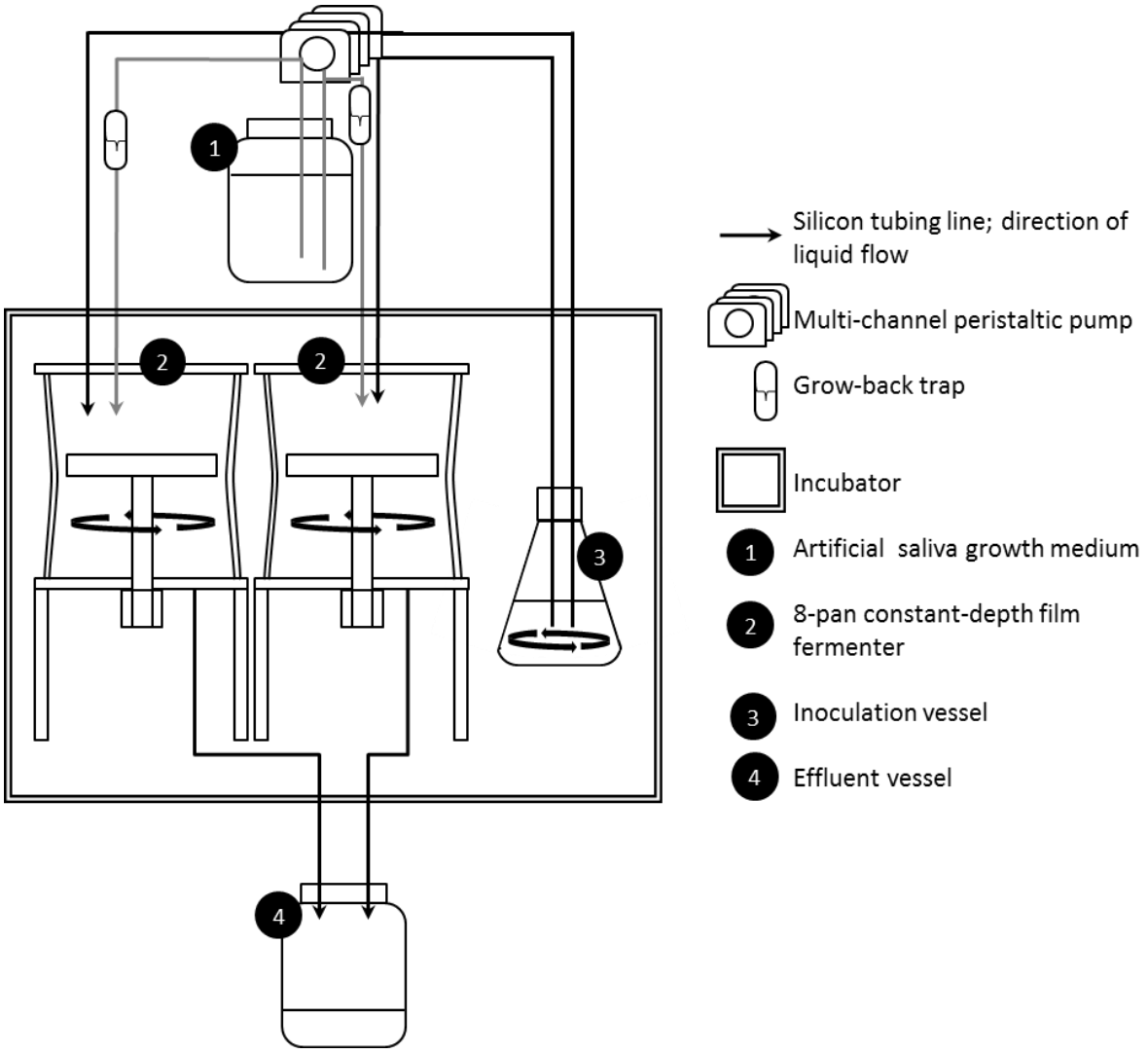


Figure 4.1. Circuit diagram, with key, of dual constant depth film fermenter biofilm model.

In a separate series of experiments two CDFFs were prepared as described above and orally representative biofilm was allowed to develop. Ten litres of 10% (w/v) (292 mM) sucrose solution was prepared, autoclaved at 116°C (1900 mBar) for 15 minutes to avoid alterations to the molecular structure of the dissolved sugars and connected to one CDFF of each dual model. 330 mL of the sucrose solution was pumped into the CDFF at 9 am, 1 pm and 5 pm daily for 30 mins according to pulsing strategies selected in previous studies (Igarashi *et al.*, 1989, 1990; Pratten and Wilson, 1999; Hope *et al.*, 2005). In one experiment sample pans were removed from both CDFFs on days 4, 6, 8, 11, 13, 15 and 18. In another, samples were taken simultaneously on days 2, 3, 5, 7 and 8.

4.2.1. Statistical Analyses

The hypothesis that differences exist between saliva pool aliquots according to members of its microbial population was investigated by one-way ANOVA, un-paired t-tests and F-tests. Sidak's multiple comparisons tests provided information on which aliquots differed according to their microbial constituent groups. The level of agreement between CDFFs exposed to the same growth conditions was examined in the constituent biofilm populations associated with dental caries (mutans streptococci, *Streptococcus* spp. and *Lactobacillus* spp.) and in the bacteria as a whole. All analysis described was undertaken to elucidate the levels of agreement between CDFFs within the dual system and when CDFFs are operated in series. **T**he Bland-Altman approach (Bland and Altman, 1986; Altman, 1991; Bland and Altman, 1999), also known as the Tukey mean difference plots, was employed to test possible agreement between bacterial counts in paired samples more rigorously. Analysis included the calculation of average difference and limits of agreement as well as graphical display of datasets as scatter plots. The theory underpinning Bland-Altman plots was extended to look for agreement rather than variance as encouraged by statisticians presently. Bias was tested using paired t-tests. The hypothesis that the microbial ecology of multispecies biofilm would differ when exposed to sucrose compared to no sucrose was investigated using an analysis of covariance (ANCOVA) model adjusting for time. Prior to all analysis normal distributions of the raw data was checked and transformed where needed and all datasets were routinely checked for outliers as described in Section 2.9.

4.3 RESULTS

4.3.1 Microbial differences in saliva pool aliquots

Figure 4.2 shows the microbial composition of five samples taken from separate aliquots of saliva pool used to inoculate the CDFF on different occasions. Sulfur producers were only determined in samples 1 and 2 while *Lactobacillus* spp. counts determined only in samples 3 – 5. One-way ANOVA tests revealed statistically significant differences between each saliva pool aliquot with regards to CFUs of total bacteria, *Streptococcus* spp., total mutans streptococci and *Lactobacillus* spp. (Table 4.1 – 4.4 respectively). Results of a two-tailed unpaired t-test revealed a statistically significant difference between numbers of sulfur producing organisms between aliquots of the saliva pool ($p < 0.0001$, $t = 81.89$, $df = 6$). An F-test evidenced no significant difference between the variances of populations the samples were derived from; endorsing the use of t-test; F (3, 3,) = 3.34, $p = 0.35$. Sidak's multiple comparison test revealed a combination of significant and non-significant differences between the microbial constituents of each aliquot of the saliva pool (Table 4.5).

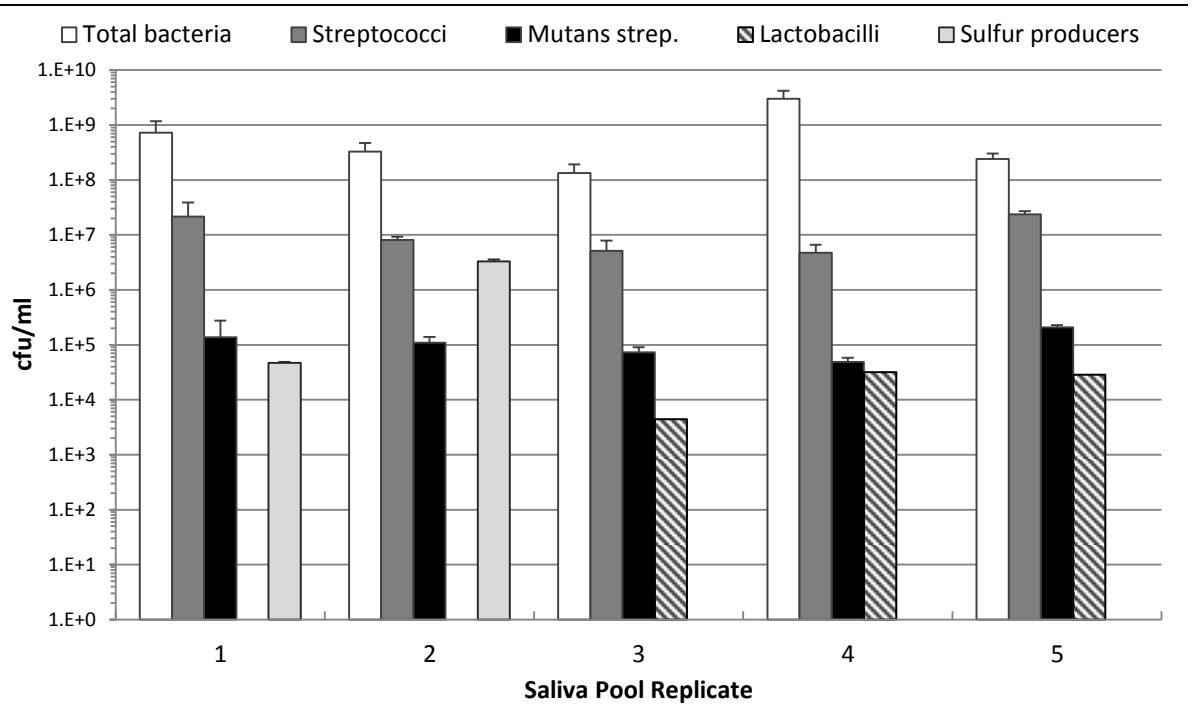


Figure 4.2. Microbial constituents of saliva pool aliquots used in inoculating different CDFF experiments. Error bars represent standard deviation, $n = 4$.

Table 4.1. One-way ANOVA results testing the statistical significance between total viable bacteria* of five saliva pool aliquots.

Source of Variation	SS	*df*	MS	F	*p*
Between Groups	4.290	4	1.070	(4, 15) = 31.5	**< 0.001**
Within Groups	0.510	15	0.034		
Total	4.800	19			

Table 4.2. One-way ANOVA results testing the statistical significance between total *Streptococcus* sp.* of five saliva pool aliquots.

Source of Variation	SS	*df*	MS	F	*p*
Between Groups	1.710	4	0.427	(4, 15) = 11.2	**0.002**
Within Groups	0.569	15	0.038		
Total	2.279	19			

Table 4.3. One-way ANOVA results testing the statistical significance between mutans streptococci group* of five saliva pool aliquots.

Source of Variation	SS	*df*	MS	F	*p*
Between Groups	0.953	4	0.238	(4, 15) = 21.4	**< 0.001**
Within Groups	0.167	15	0.011		
Total	1.12	19			

Table 4.4. One-way ANOVA results testing the statistical significance between *Lactobacillus* sp.* of five saliva pool aliquots.

Source of Variation	SS	*df*	MS	F	*p*
Between Groups	0.128	2	0.0639	(2, 9) = 13.6	**0.0019**
Within Groups	0.042	9	0.0047		
Total	0.170	11			

Sum of squares, SS; degrees of freedom, df; mean square, MS;
F = (MS between groups)/(MS within groups). *$\log_{10}$ transformed prior to analysis.
bold font emphasises statistical significance.

Table 4.5. Results of multiple comparisons testing the statistical significance between microbial counts from five aliquots of saliva pool inoculum

Pool Aliq.	Total bacteria*			*Streptococcus* spp.*			Mutans strep.*			*Lactobacillus* spp.*		
	MD	±CI$^{95\%}$	*p*	MD	±CI$^{95\%}$	*p*	MD	±CI$^{95\%}$	*p*	MD	±CI$^{95\%}$	*p*
1 vs 2	0.315	0.112, 0.742	0.2539	0.335	-0.116, 0.786	0.2475	0.0975	-0.147, 0.342	0.9059	-	-	-
1 vs 3	0.710	0.283, 1.14	**0.0007**	0.568	0.116, 1.02	**0.0091**	0.272	0.028, 0.517	**0.0231**	-	-	-
1 vs 4	-0.643	-1.070, 0.216	**0.0018**	0.585	0.134, 1.04	**0.0070**	0.442	0.198, 0.687	**0.0003**	-	-	-
1 vs 5	0.435	0.008, 0.862	**0.0441**	-0.132	-0.584, 0.319	0.9868	-0.190	-0.434, 0.0541	0.2013	-	-	-
2 vs 3	0.395	0.0318, 0.822	0.0812	0.232	-0.219, 0.684	0.6957	0.175	-0.069, 0.419	0.2850	-	-	-
2 vs 4	-0.958	-1.380, 0.531	**< 0.001**	0.250	-0.201, 0.701	0.6090	0.345	0.101, 0.589	**0.0033**	-	-	-
2 vs 5	0.120	-0.307, 0.547	0.9904	-0.468	-0.919, 0.0163	**0.0394**	-0.288	-0.532, -0.043	**0.0154**	-	-	-
3 vs 4	-1.350	-1.78, -0.926	**< 0.001**	0.0175	-0.434, 0.469	> 0.9999	0.170	-0.074, 0.414	0.3182	-0.135	-0.276, 0.0065	0.0619
3 vs 5	-0.275	-0.702, 0.152	0.4143	-0.700	-1.15, 0.249	**0.0014**	-0.463	-0.707, -0.218	**0.0002**	0.118	-0.024, 0.259	0.1101
4 vs 5	1.08	0.651, 1.50	**< 0.001**	-0.718	-1.17, 0.266	**0.0011**	-0.633	-0.877, -0.388	< **0.0001**	0.252	0.111, 0.394	**0.0017**

Mean difference, MD; upper and lower confidence intervals, ±CI$^{95\%}$

*$\log_{10}$ transformed prior to analysis; **bold** font denotes difference is statistically significant

4.3.2 Biofilm formation and growth

The dual CDFF system developed in this study and CDFFs when operated on separate occasions supported the growth of multispecies biofilm in which *Streptococcus* spp., mutans streptococci and *Lactobacillus* spp. were detectable. The biofilm community reached a pseudo-steady state by day 4 (Figure 4.3). In relation to the total counts of bacteria on nonselective blood agar, the proportions of *Streptococcus* spp. ranged from 1.904 to 14.432%, mutans streptococci from 0.003 to 0.004% and *Lactobacillus* spp. from 0.650 to 1.036%. Numbers of *Streptococcus* spp. were present at relatively steady proportions throughout each experiment and viable counts of the constituent groups of interest provide evidence of succession over time. Bacteria of the mutans streptococci group were most numerous early in the dual

CDFF run before diminishing to undetectable numbers as *Lactobacillus* spp. proportions began to increase. *Lactobacillus* spp. counts plateaued as steady state was reached.

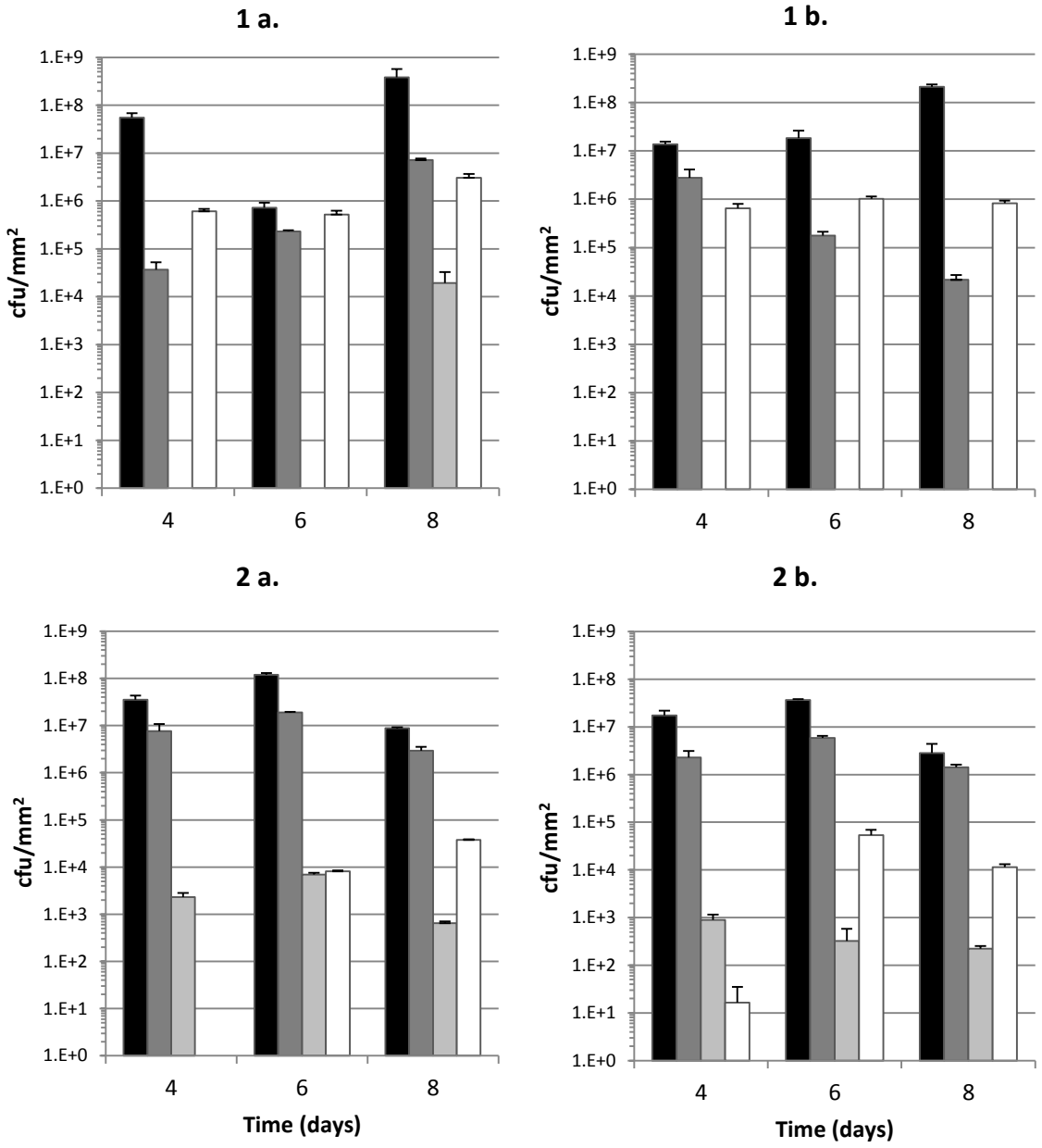


Figure 4.3. Microbial composition of multispecies oral biofilm grown under identical growth conditions using the novel dual CDFF model (a and b) on two separate occasions (1 and 2). Total viable bacteria, ■; total streptococci, ■; mutans streptococci, □; total lactobacilli, □. Error bars represent standard deviation, n = 4.

4.3.3 Explorative analysis of agreement

The level of agreement between experiments performed on different occasions and between those undertaken in parallel within the novel dual CDFF model was investigated. There was no evidence of systematic bias between pairs of samples for any of the microbial groups when CDFFs of the dual model were compared. For pairs of samples run on separate occasions, there were statistically significant differences between pairs for *Streptococcus* spp., mutans streptococci and *Lactobacillus* spp., suggesting systematic bias between runs (Table 4.6). Coefficients$_{95\%}$ of agreement were lower, therefore less variable, in the dual model than when run in series for total counts of bacteria (1.238 vs 2.124), *Lactobacillus* spp. (0.517 vs 1.431) and mutans streptococci (2.817 vs 3.864). A similar trend was observed in the limits of agreement$_{95\%}$ (Table 4.6). Bland-Altman plots for total count for each microbial group (Figure 4.4) show the differences between $\log_{10}$ viable microbial counts from pairs of samples, plotted against their respective averages.

Table 4.6. Analysis of agreement between log viable bacterial counts of oral biofilm in CDFFs operated in parallel within the dual model (*d*) or in series (*s*).

	Total bacteria*		*Streptococcus* spp*		Mutans streptococci*		*Lactobacillus* spp.*	
	d	*s*	*d*	*s*	*d*	*s*	*d*	*s*
Minimum difference	0.058	-0.11	-0.035	0.085	0.000	-1.937	-0.029	0.985
Maximum difference	-1.406	-2.21	2.522	-2.676	4.290	-3.844	-0.819	5.820
Mean difference	0.173	0.181	0.342	-1.287	0.722	-2.372	-0.058	2.874
Standard deviation (SD)	0.619	1.391	1.142	1.131	1.409	1.917	0.517	1.794
Coefficient $_{95\%}$ of agreement	1.238	2.781	2.285	2.262	2.817	3.864	1.035	3.589
Limits $_{95\%}$ of agreement	-1.065, +1.410	-2.600, +2.963	-1.942, +2.627	-3.549, +0.975	-2.095, +3.539	-6.206, +1.462	-1.092, +0.977	-0.715, +6.463
P-value (paired t-test)	0.426	0.660	0.396	**0.002**	0.389	**<0.001**	0.162	**0.002**

CDFF experiment: *d*, concurrent (n=9); *s*, in series (n=12); *$\log_{10}$ transformed bacterial counts

bold font denotes difference is statistically significant

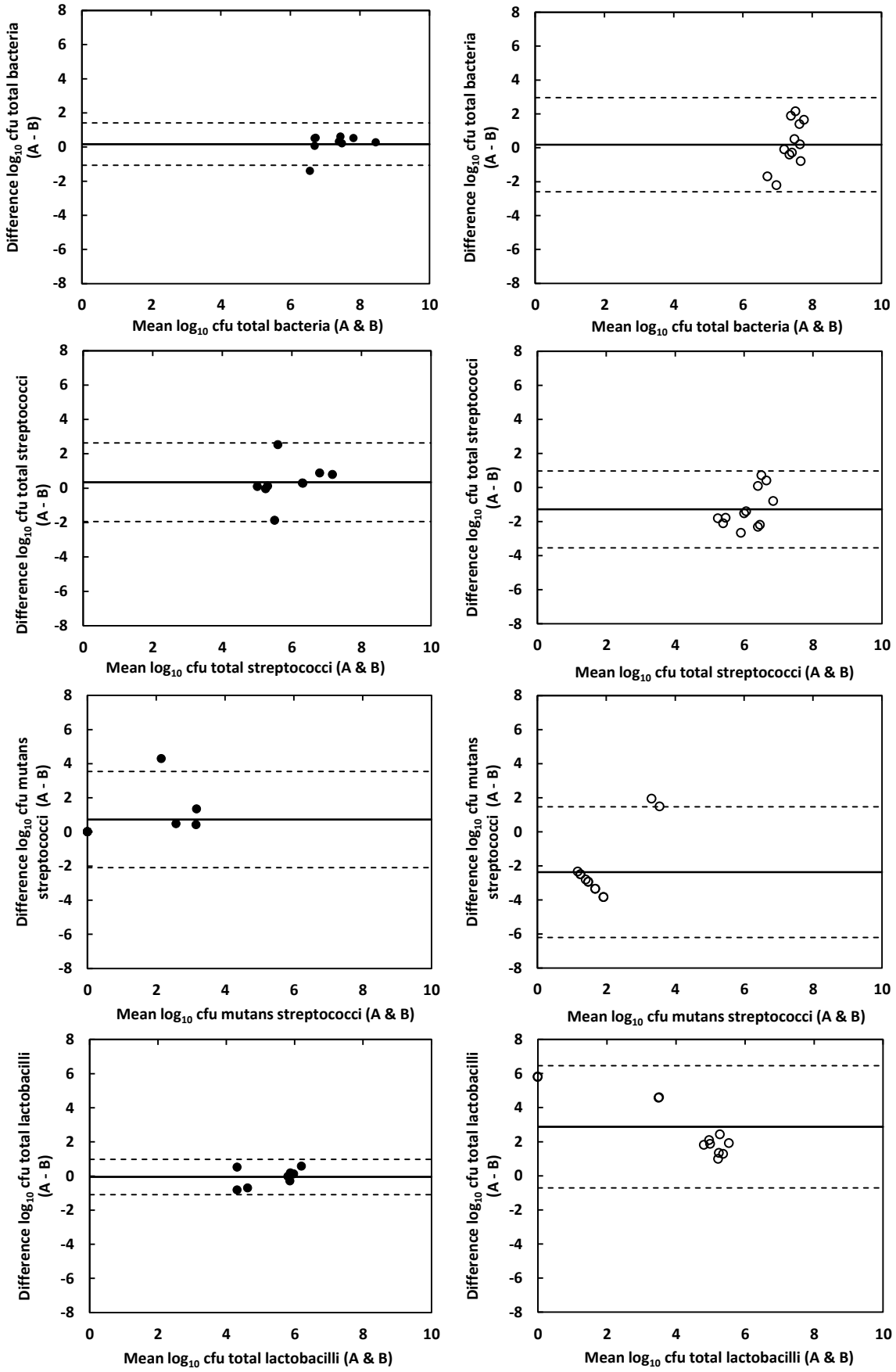


Figure 4.4. Bland – Altman plots illustrating levels of agreement between microbial counts in two CDFFs operated concurrently (●) or in series (○). Solid line represents the mean difference, while dashed lines depict upper and lower 95% limits of agreement (mean ± 2SD).

4.3.4 Preliminary studies of dual model in assessing the effect of sucrose on biofilm microbial ecology

Figure 4.5 shows the microbial composition of multispecies oral biofilm when pulsed with 10% (292 mM) sucrose, three times daily or with no sucrose as a control. Datasets from two experiments are included on each graph; early biofilm growth from day 2 – 8 represented by long dash lines and solid and short dash lines represents bacterial numbers from day 4 – 18. Counts were lower in both total viable bacteria ($3.84 \times 10^{10} \pm 5.12 \times 10^{10}$ SD cfu mm^{-2} vs $1.51 \times 10^{11} \pm 3.16 \times 10^{11}$ SD cfu mm^{-2}; average of day 4 – 18 counts, $n = 7$) and in total *Streptococcus* spp. ($4.20 \times 10^{5} \pm 9.13 \times 10^{5}$ SD cfu mm^{-2} vs $3.78 \times 10^{7} \pm 4.14 \times 10^{7}$ SD cfu mm^{-2}; average of day 4 – 18 counts, $n = 7$) in biofilm exposed to 10% (292 mM) sucrose compared to the control biofilm whilst mutans streptococci numbers were similar in both the presence ($9.97 \times 10^{5} \pm 1.09 \times 10^{6}$ SD cfu mm^{-2}; average of day 4 – 18 counts, $n = 7$) and absence of sucrose ($6.85 \times 10^{7} \pm 1.62 \times 10^{8}$ SD cfu mm^{-2}; average of day 4 – 18 counts, $n = 7$). Prior to day 5 total viable counts were similar in each condition ($2.90 \times 10^{9} \pm 5.03 \times 10^{9}$ SD cfu mm^{-2} in sucrose versus $1.72 \times 10^{9} \pm 2.97 \times 10^{9}$ SD cfu mm^{-2} in no sucrose; both averages of day 2 – 5 counts, $n = 3$). However, total *Streptococcus* spp. were considerably lower, and mutans streptococci counts were moderately lower, in sucrose exposed biofilm. $2.81 \times 10^{5} \pm 4.86 \times 10^{5}$ SD cfu mm^{-2} *Streptococcus* spp. were present in sucrose compared to $2.58 \times 10^{7} \pm 4.47 \times 10^{7}$ SD cfu mm^{-2} without sucrose; and $5.04 \times 10^{6} \pm 9.65 \times 10^{6}$ SD cfu mm^{-2} mutans streptococci in sucrose compared to $8.06 \times 10^{7} \pm 1.53 \times 10^{8}$ SD cfu mm^{-2} in the control biofilm (all are averages of day 2 – 8 counts, $n = 5$). By day 8 *Lactobacillus* spp. growth in sucrose exposed biofilm increased markedly (22.1 ± 3.91 SD cfu mm^{-2}, $n = 4$, compared to 1.38 ± 3.91 SD cfu mm^{-2}, $n = 4$, at day 8 in control biofilm) while in the absence of additional sucrose *Lactobacillus* spp. were only detectable at day 6 (27.1 ± 78.2 SD cfu mm^{-2}, $n = 4$) and day 18 (33.2 ± 5.47 SD cfu mm^{-2}, $n = 4$).

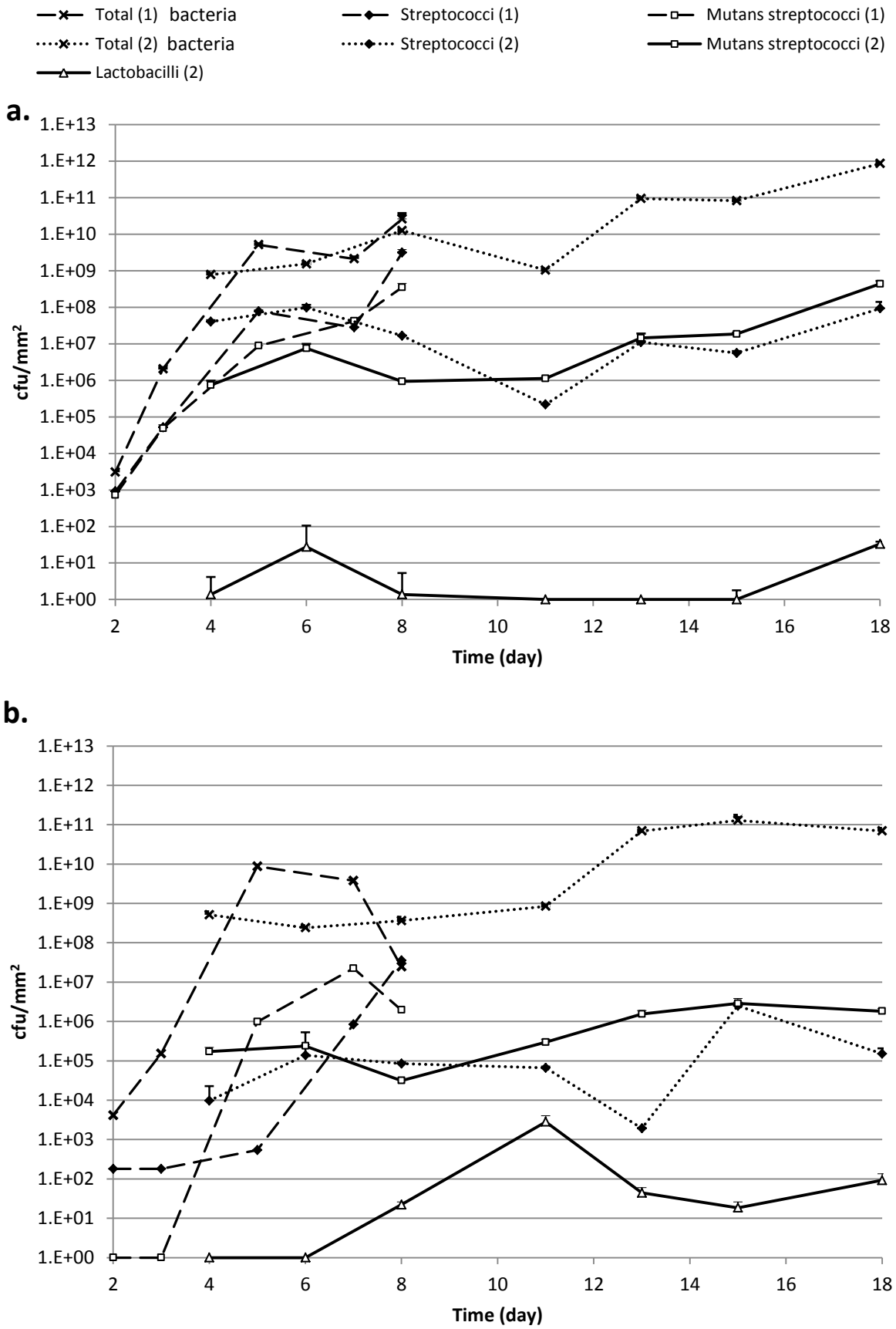


Figure 4.5. Microbial composition of multispecies oral biofilm grown on hydroxyapatite exposed to (a) no sucrose or (b) sucrose 3 times daily. Graphs include results of two experiments. Error bars represent standard deviation, $n = 4$.

Tables 4.7 – 4.10 and 4.11 – 4.13 summarise the results of statistical testing for the factors of time and sucrose addition in the 18 day experiment and in the 8 day experiment respectively. ANCOVA testing revealed that differences over time in all CDFFs of each experiment were statistically significant ($p < 0.001$) supporting the interpretation that bacterial changes were occurring over time. When looking at the possible effect of sucrose on the multispecies biofilm, total viable bacteria were not statistically different in either the 18-day ($p = 0.082$) or 8-day ($p = 0.745$) experiments. Total *Streptococcus* spp. significantly differed with sucrose addition only in the 18-day experiment ($p = 0.001$) but this statistically significant difference was not replicated in the model of early biofilm formation ($p = 0.149$). Whereas, differences in numbers of mutans streptococci and *Lactobacillus* spp. exposed to sucrose or not, over 18 days were not statistically significant ($p = 0.069$ and 0.927 respectively). However, statistically significant differences were found when comparing mutans streptococci in biofilm exposed to sucrose compared to the unpulsed control in the 8-day long experiment ($p = 0.003$).

Table 4.7. Results of ANCOVA testing statistical significance of time and sucrose addition in growth of **total viable bacteria*** over **18 days** exposed to **no or 10% sucrose** three times daily.

Source of Variation	SSIII	*df*	MS	F	*p*
Sucrose	0.854	2	0.854	3.155	0.082
Time	48.776	1	48.776	180.208	**< 0.001**
Sucrose x time	0.019	1	0.019	0.070	0.792

R Squared = 0.798 (Adjusted R Squared = 0.787)

Table 4.8. Results of ANCOVA testing statistical significance of time and sucrose addition in growth of **total *Streptococcus* spp.*** over **18 days** exposed to **no or 10% sucrose** three times daily.

Source of Variation	SSIII	*df*	MS	F	*p*
Sucrose	85.123	2	85.123	44.598	**< 0.001**
Time	16.352	1	16.352	8.567	**0.005**
Sucrose x time	25.188	1	25.188	13.197	**0.001**

R Squared = 0.640 (Adjusted R Squared = 0.619)

Table 4.9. Results of ANCOVA testing statistical significance of time and sucrose addition in growth of **mutans streptococci*** over **18 days** exposed to **no or 10% sucrose** three times daily.

Source of Variation	SSIII	*df*	MS	F	*p*
Sucrose	0.858	2	0.858	3.441	0.069
Time	21.486	1	21.486	86.201	**< 0.001**
Sucrose x time	0.863	1	0.863	3.463	0.068

R Squared = 0.765 (Adjusted R Squared = 0.752)

Table 4.10. Results of ANCOVA testing statistical significance of time and sucrose addition in growth of **total *Lactobacillus* spp.*** over **18 days** exposed to **no or 10% sucrose** three times daily

Source of Variation	SSIII	*df*	MS	F	*p*
Sucrose	0.005	2	0.005	0.008	0.927
Time	9.687	1	9.687	15.050	**< 0.001**
Sucrose x time	2.569	1	2.569	3.991	0.051

R Squared = 0.449 (Adjusted R Squared = 0.417)

Type III sum of squares, SSIII; degrees of freedom, *df*; mean square, MS; F = (MS between groups)/(MS within groups). *$\log_{10}$ transformed prior to analysis. **bold** font denotes difference is statistically significant.

Table 4.11. Results of ANCOVA testing statistical significance of time and sucrose addition in growth of **total viable bacteria*** over **8 days** exposed to **no or 10% sucrose** three times daily.

Source of Variation	SSIII	*df*	MS	F	*p*
Sucrose	0.282	2	0.282	0.110	0.745
Time	84.582	1	84.582	32.879	**< 0.001**
Sucrose x time	1.703	1	1.703	0.662	0.428

R Squared = 0.683 (Adjusted R Squared = 0.624)

Table 4.12. Results of ANCOVA testing statistical significance of time and sucrose addition in growth of **total *Streptococcus* spp.*** over **8 days** exposed to **no or 10% sucrose** three times daily.

Source of Variation	SSIII	*df*	MS	F	*p*
Sucrose	1.484	2	1.484	2.302	0.149
Time	95.712	1	95.712	148.441	**< 0.001**
Sucrose x time	0.003	1	0.003	0.004	0.951

R Squared = 0.911 (Adjusted R Squared = 0.894)

Table 4.13. Results of ANCOVA testing statistical significance of time and sucrose addition in growth of **mutans streptococci*** over **8 days** exposed to **no or 10% sucrose** three times daily.

Source of Variation	SSIII	*df*	MS	F	*p*
Sucrose	15.517	2	15.517	12.001	**0.003**
Time	122.822	1	122.822	94.995	**< 0.001**
Sucrose x time	4.330	1	4.330	3.349	0.086

R Squared = 0.880 (Adjusted R Squared = 0.857)

Type III sum of squares, SSIII; degrees of freedom, *df*; mean square, MS; F = (MS between groups)/(MS within groups). *$\log_{10}$ transformed prior to analysis. **bold** font denotes difference is statistically significant.

4.4 DISCUSSION

A reduced level of variability was detected between biofilm produced using the dual CDFF model compared to biofilm from different CDFF experiments and the effect of sucrose exposure on multispecies biofilm within the dual model was demonstrated. Comparing results from CDFFs operated on separate occasions can give rise to significant variation even if the inoculum is defined (Kinniment *et al.*, 1996b) or if microcosm from the same source is used (Pratten *et al.*, 1998a). The question of reproducibility between individual biofilm experiments has been discussed extensively in the past. Within the defined nine-species biofilm model described by Kinniment *et al* (1996b) a significant ($p = 0.01$) variation between runs was reported and essentially it was found to be difficult to replicate the defined community within the biofilm. When using mixed bacterial populations as an inoculum, differences between compositions of biofilms have been described despite inoculum being derived from the same source (Pratten *et al.*, 1998a). These variations have been attributed to slight unavoidable differences between aspects of CDFF operation worsened by the potential effects of heterogeneity in the inoculum (Ledder *et al.*, 2006) and whether unculturable microorganisms are present within it (Pratten *et al.*, 2003). Despite these reported limitations, the CDFF remains a versatile method for the production of reproducible aliquots of oral biofilm (Pratten and Wilson, 1999). The variability between CDFF experiments has the most pertinent implications when separate CDFF runs are to be compared with each other; for example, when comparing the effects of experimental conditions on biofilm formation, microbial composition and in a caries model; the state of the dental hard tissue substratum. Therefore, reducing the variability within the design of a study will enhance its detection limits; an improvement of particular use when assessing the effects of test agents or other changes in variables of interest which can often be minute but significant.

4.4.1 Reducing the variability between CDFF experiments when modelling oral biofilm

The dual CDFF model improves upon the design of the oscillating "split CDFF" model which also improved reproducibility within studies and reduced the impact of minor variations (Hoogenkamp *et al.*, 2003; Deng *et al.*, 2005). The split model only holds eleven useable sample pans; meaning five are exposed to agents on one side of the fermenter and six are on the other whilst the dual model provides sixteen sampling opportunities; eight for each test condition. Additionally, though it was shown that there was no carry-over of bacteria or agents between the two sides of the split fermenter, the complete separation of the two units within the dual CDFF model ensured no cross-over of bacteria or materials between the two units. The only physical connections between the two CDFFs of the dual model were at the point of medium ingress, which was protected by grow-back traps, and in the effluent, where the two inputs are separated by 15 mm. The typical flow rate of artificial saliva into a CDFF was nominally set at 0.5 – 0.72 ml min^{-1} (Pratten, 2007) to mimic the mean flow rate of saliva in man (Lamb *et al.*, 1991; Guyton *et al.*, 1997). The flow of artificial saliva over the sample pans of the smaller CDFF was proportionally adjusted to account for eight rather than fifteen sample pans (0.72 ml min^{-1} × $^{8}/_{15}$ = 0.38 ml min^{-1}) in order to maintain the same flow relative to the number of sample pans present. The flow rate of the inoculum into the two CDFFs remained 0.72 ml min^{-1} since the growth kinetics within the inoculation vessel and its standard delivery into the CDFF was considered important.

The theory underpinning the Bland-Altman approach was considered appropriate for testing for similarity between the biofilms developed either on separate occasions or within the dual model. Originally developed as a statistical means to measure the level of agreement that may exist between two methods of measurement, it has been extensively used in the area of method/technique comparison and validation (Dewitte

et al., 2002; Müller and Behbehani, 2005; Moses *et al.*, 2009). In the past, the product-moment correlation coefficient (*r*) has been given in many studies, as an indicator of agreement between two variables; sometimes together with regression analysis. It has been shown that this is an inappropriate approach when testing for agreement (Bland and Altman, 1986; Altman, 1991; Bland and Altman, 1999). These authors stress that correlation (*r*) measures the strength of the relation between two variables and cannot indicate how closely these agree. Furthermore it should be noted that many investigators regard a large *r* value and small *p* value as enough to indicate a good degree of agreement no matter the pattern of scatter. Testing for significant difference between variables using a paired *t*-test is another incorrect route to employ, as this method will be less likely to give a significant result the worse the agreement is. Bland and Altman suggest that the appropriate analysis of comparison studies should simply be based on the mean and standard deviation of the between variable differences.

Although the mean differences in viable counts of bacteria provide a good descriptive of the difference between biofilms produced within CDFFs, the most objective measure is to deduce the degree of variability observed in each type of model – in this case either dual or in series CDFFs. Standard deviation was much lower when comparing CDFFs run concurrently within the dual CDFF design (0.619 $\log_{10}$ cfu mm^{-2}) than with those compared from separate occasions (1.391 $\log_{10}$ cfu mm^{-2}). Therefore the new dual CDFF design minimises the degree of difference and variability between CDFFs to be compared. A certain degree of variability, or lack of agreement, is inevitable when comparing two microbiological growth experiments in a CDFF. This is due in part to the inherent complexity in their operation, but more importantly to the dynamic multispecies ecosystem that develops into a pseudo steady-state microbial biofilm ecosystem which undergoes periodic sloughing events.

Furthermore, the microcosms used to inoculate the systems contain a diverse range of constituents sensitive to even slight differences though the results confirm that the dual model minimises the impact of the inherent heterogeneity of the inoculum.

Additionally, slight differences in the mechanisms of a particular CDFF e.g. the angle and condition of the scraper blades could cause differences in the biofilm architecture; ensuring this parameter is standardised would lead to greater consistency between biofilm samples. It is possible that minor defects in the edge of a scraper blade could snag and mean biofilms are removed entirely from the substratum since the bond between constituents of a biofilm can be stronger than the biofilm's association with the underlying substratum. Should this occur, bacterial numbers would be affected at specific time points due to improper biofilm formation. The proximity of the inlet ports to scraper blades and the direction of turntable motion could have additional impact. Therefore the methodology would benefit from the standardisation of which particular input port is used for each incoming liquid. Nonetheless, these parameters can be optimised easily for future work and the use of the Bland-Altman approach offers a good method of analysis for elucidating the differences between biofilms produced in each CDFF and the variability therein. In conclusion, operating CDFFs concurrently, in the 'dual' modality minimises both the degree of difference and variability, and reduces the likelihood of systematic bias between CDFF experiments designed to compare the effects of potential variables, such as the addition of experimental agents.

4.4.2 Growth of bacterial members of multispecies biofilm exposed to 10% sucrose thrice daily

Pulsing multispecies biofilm three times daily with 10% sucrose appeared to both modulate and encourage the growth of total biofilm bacteria grown over 18 and 8 days respectively, but differences when compared to biofilm exposed to no additional

sucrose were found not to be statistically significant over both time courses. Since, in the current study, complex multispecies biofilm were developed it is understandable that as numbers of specific bacterial groups are changing the sum remains steady over time. Microorganisms that were not specifically enumerated in the design of this study have the potential to grow to take the place of others as they perish. Complex microbial interactions ensure the space and resources available are fully utilised by the diverse members of the microbial community within dental plaque (Connell and Slatyer, 1977; Fredrickson, 1977; Baier, 1984; Wahl, 1989; Marsh and Bradshaw, 1995). This is further achieved since the CDFF model system maintains biofilm at a constant depth, constantly applying the limitation of volume growth, as in the *in vivo* situation. Though proportions of *Streptococcus* spp. and mutans streptococci were supressed in the presence of 10% sucrose, increases in *Lactobacillus* spp. counts suggest that the biofilm was more acidic under these conditions compared to when additional sucrose is lacking. *Lactobacillus* spp. are highly aciduric bacteria often associated with caries due to their ability to metabolise and survive in acidic conditions compared to other oral bacteria. The same can be claimed for mutans streptococci also. The proton-translocating ATPase developed by members of both bacterial groups, means they are capable of maintaining their intracellular pH between 0.5 and 1 unit higher than the extracellular environment (Bowen, 2002). *L. casei* was observed as more resistant to environmental acidification with gross membrane damage only evident at pH values lower than 3.0 whereas it appears *S. mutans*, a moderately aciduric bacteria, does not possess barriers to small molecules at pH values significantly below 4.0 (Bender *et al.*, 1986). In this respect the membrane ATPases constitute major workhorses of acid tolerance for oral streptococci in particular since *S. mutans* can operate glycolytic functions at pH as low as 4.0, functions which directly relate to their cariogenicity. Therefore these mechanisms inherently protect acid-sensitive glycolytic enzymes in the cytoplasm by moving protons out of the cell through the ATPase components of the cell membrane. The

final result is that glycolysis, and so cariogenicity, is more aciduric than functions involved in cell growth (Bender *et al.*, 1986). This selective advantage is key in their survival within acidic environments, such as dental plaque post sucrose exposure, and this has been demonstrated in the present study in the growth trends of *Lactobacillus* spp. specifically. Nonetheless, minimal pH in dental plaque is not often lower than pH 4.0 (Schachtele and Jensen, 1982), but subtleties of the diverse microenvironments within dental plaque may be the source of persistence by bacteria well adapted compared to those less well adapted to conditions of acidification.

Although mutans streptococci are also able to preferentially grow in the presence of acid and sucrose compared to other oral streptococcal bacteria (Bowen, 2002), viable bacterial counts of this group were significantly lower in biofilm exposed to 10% sucrose over 8 days compared to their numbers in biofilm not exposed to sucrose. However, no statistically significant differences were observed over the 18 day experiment. Since growth was less limited once biofilm was established, results suggest the presence of sucrose selected for the proliferation of mutans streptococci adapted to the rapid transport and metabolism of sucrose (Ajdic *et al.*, 2002; Ajdic and Pham, 2007; Ajdic and Chen, 2013). These findings are in line with previous work indicating that different strains of *S. mutans* possess differential abilities for acid tolerance and that adhesion to surfaces plays an important role in the acid tolerance of cells in biofilm (Welin-Neilands and Svensäter, 2007). It can also be theorised that at early stages mutans streptococci are concertedly forming EPS from the sucrose substrate, a key feature of this bacterial group, which results in less physical volume available for bacterial cells. Elsewhere, it was shown that the frequency of sucrose exposure and its concentration appear to increase EPS concentration in biofilm matrices, lowering pH during fasting periods and enhancing demineralisation (Cury *et al.*, 1997; Cury *et al.*, 2000; Ribeiro *et al.*, 2005; Aires *et al.*, 2006). The ability of *S. mutans* to produce large amounts of extraceullar, sticky glucans has been considered

a key aspect of plaque formation (Gibbons and Nygaard, 1968; Gibbons, 1989). Furthermore, sucrose supplemented biofilms were more resistant to physical disruption by electric toothbrushes in an *in vitro* model and this is likely due to alterations in the biophysical structure associated with increased EPS formation in the presence of sucrose (Hope *et al.*, 2005). In essence, it is the behaviour of microbial elements of biofilm and less the proportions or presence of particular bacterial species that is key in ascertaining their specific role in determining the cariogenicity of biofilm. This is supported in studies of a wide range of cariogenic features of dental plaque bacteria such as in acid formation, sugar metabolism and, in EPS formation. An example of the latter is in studies revealing that the demineralisation of enamel was greatest when artificial biofilm analogous to dental plaque consisted of 95% EPS and only 5% *S.mutans* than other ratios with higher proportions of bacteria (Zero *et al.*, 1986a). Furthermore, the recent identification of a specific PTS expressed in biofilm of *S. mutans* fed with sucrose possibly involved in the transport and metabolism of carbohydrates within EPS, provides further impetus for *S. mutans* to preferentially produce EPS as a selective advantage (Ajdic and Chen, 2013).

Numbers of *Streptococcus* spp. were significantly lower in biofilm exposed to sucrose over 18 days than in its absence whereas no statistically significant difference between bacterial numbers was observed over the 8 day experiment. This is likely due to the inability of some streptococcal species to survive and persist under acidic conditions and reflects that it is likely that younger biofilm was less acidic and biofilm became more acidic as it matured. For example, *S. sanguinis* possesses relatively low acid tolerance with the optimum pH for its ATPase found to be 7.5, compared to 7.0, 6.0 and 5.0 for *S salivarius, S. mutans* and *L. casei* respectively (Bender *et al.*, 1986). *S. salivarius* exhibited intermediate acid tolerance with least permeability to protons following acid pulse at around pH 6.0 and average halftime for pH equilibration across the cell membrane at around 6.6 mins compared to values for *S. sanguinis* (pH

7.0 and 8.2 minutes) and *S. mutans* (pH 5.0 and 12 mins) (Bender *et al.*, 1986). It also appears that *S. salivarius* is less aciduric in dense glycolysing suspensions than in growing cultures since this organism was able to grow in complex media at pH values as low as the minimum growth value for *S. mutans* (Bender *et al.*, 1986).

Pulsing with 330 ml 10% (292 mM) sucrose thrice daily equates to the total daily sucrose intake in adults in the UK on average (Burt, 1993), directly models the consumption of sucrose-rich soft drinks (Lagerlof *et al.*, 1984; Lindfors and Lagerlof, 1988) and has been used to model cariogenic challenges in the past (Igarashi *et al.*, 1989, 1990). Previous CDFF experiments that employed this level and frequency of sucrose supplementation were able to demonstrate a decreased susceptibility to amine fluorides (Embleton *et al.*, 1998) and a marked change in biofilm architectural structure with 85% streptococci (Pratten and Wilson, 1999) in biofilm exposed to these conditions, an observation replicated in the present study. This preliminary study has demonstrated the application of the novel dual CDFF model in comparatively modelling the effects of supplementation with dietary nutrients to mimic factors affecting dental plaque *in vivo*. The findings of this study further demonstrate key aspects of the known effect of sucrose on oral bacteria.

4.5 CONCLUSIONS

The present study demonstrates the development of a novel approach in studying biofilm using the CDFF as a basis for subsequent studies to elucidate the effects of fluoride in caries control. Operating the CDFF systems identically within the dual modality and on separate occasions enabled the evaluation of the ability of the proposed dual model to minimise variability in biofilms to be compared directly. The experimental findings indicate that operating CDFFs concurrently, within the dual model reduces both the degree of difference and variability in the microbiological

aspects of biologically relevant multispecies oral biofilm. Less variability was observed in the concurrent model than when CDFFs were run in series for (log transformed) total bacteria (1.24 vs 2.12), *Lactobacillus* spp. (0.52 vs 1.43) and mutans streptococci (2.82 vs 3.86) according to the 95% coefficients of agreement. The degree of difference was also lower for all bacterial groups compared within the dual design than those run separately. This experimental approach also reduces the likelihood of systematic bias between CDFF experiments designed to compare the effects of potential variables, such as the addition of experimental agents.

A preliminary study elucidated the effects of 10% sucrose on the microbial ecology of multispecies biofilm in direct comparison to non-sucrose exposed biofilm within the novel dual model. The findings were in line with the known effects of sucrose on oral bacteria as well as the known behaviour of biofilm in response to environmental stresses. Sucrose increased the acidity of biofilm as indicated by the proliferation of *Lactobacillus* spp. in its presence. Though the quantities of mutans streptococci were significantly suppressed in 10% sucrose over an 8 day period, there were no statistically significant differences in their numbers over an 18 day period. Since growth was less limited once biofilm was established, it is possible that the presence of sucrose selected for the proliferation of mutans streptococci adapted to the rapid transport and metabolism of sucrose. Whereas, low bacterial counts in the early stages may be indicative of the formation of EPS from the sucrose substrate by this bacterial group which results in less physical volume available for bacterial cells. This is in line with previous reports that the concentrations of sucrose and the frequency of exposure appear to increase EPS concentration in biofilm matrices. A useful future study would be to investigate identical conditions for the biofilm mediated formation of caries in enamel and dentine within the dual model and on separate occasions, however it may be a costly and futile use of resources. The

current findings indicate that the dual model will be optimal compared to CDFFs run sequentially in comparative studies involving dental tissue substrata particularly when the expected effects of an agent on mineralisation are subtle. Needless to say, caries is a biofilm mediated phenomena and the design of the present study goes some way in addressing the previously reported issues of variability in CDFF models. Refinement of the model in this way would no doubt favourably impact the comparability of caries studies. In conclusion, this model should prove useful in dental plaque research and in studies of plaque related diseases by providing a more scientifically rigorous basis for investigating the effects of such clinically important factors as diet and the use of anti-caries therapies.

Chapter 5 Modelling the effects of continuous fluoride exposure in a biological caries model

5.1 INTRODUCTION

As the main active ingredient in currently recommended oral hygiene procedures, fluoride is recognised as responsible for the significant decline in caries observed worldwide (Bratthall *et al.*, 1996) and there is strong evidence to suggest that the primary mode of action is the result of its topical activity in the oral environment (Ogaard *et al.*, 1988; Featherstone *et al.*, 1990; ten Cate, 1990). The development of fluoride release methods has garnered considerable interest. A fluoride-delivery system capable of supplying small amounts of fluoride continuously throughout the day, ensuring elevated plaque fluoride levels may constitute a prime caries preventative measure. Though it has been well established that the primary control of caries lesion development by fluoride is by its effect on de- and remineralisation at the interface of the tooth and oral fluids (Featherstone *et al.*, 1990; ten Cate, 1990) its role in effecting biofilm bacteria has also been demonstrated extensively (see Section 1.5.3). The concentration of fluoride required to inhibit the proliferation of oral microorganisms is much higher than is needed to influence the caries process in dental hard tissues. However small, its effects on acid production and glucan synthesis mean it remains worthwhile to investigate the effects of fluoride in the context of complex dental plaque microbial communities since sub-lethal concentrations of fluoride have the capacity to influence community succession (Marsh *et al.*, 2011), disturb the ecological balance within disease-states (Marsh and Bradshaw, 1997; Marsh, 2003b; Duckworth, 2013) and interrupt cariogenic features of dental plaque to form biofilm communities with cumulatively altered phenotype characteristics. While higher concentrations of fluoride appear capable of penetrating further into biofilm (Watson *et al.*, 2005; Pandit *et al.*, 2013), of reducing the growth of

a range of oral bacteria (Bibby and van Kesteren, 1940; van Loveren *et al.*, 1987; Kawashima *et al.*, 2013) and disrupting the structural integrity of their biofilm (Pandit *et al.*, 2013). It is also promising that bacterial sensitivity greatly increases as biofilm pH falls (Shiota, 1956; Jenkins, 1959), such as due to the production of acids in response to increased substrate availability when foods are consumed. To date, the application of CDFF model systems to simultaneously investigate the effect of continuous fluoride exposure on biologically relevant biofilm and caries has been relatively limited. So far, studies have elucidated the effects of 9.5 and 19 ppm fluoride on a nine-species defined consortium biofilm alone (Hoogenkamp *et al.*, 2003) and of 135 ppm fluoride on the viability and acid production of single species *S. mutans* biofilm grown in a CDFF model on dentine (Deng *et al.*, 2005). Nevertheless, none model the effects of sub-ppm and high concentrations of fluoride on multispecies biofilm and enamel caries.

The role of low levels of background fluoride in the prevention of caries has been extensively reviewed (Featherstone, 1999). Evidence shows that low but slightly elevated levels of fluoride in saliva and plaque help prevent and reverse caries by inhibiting demineralisation and enhancing remineralisation. Clinical studies, conducted in the late 1980s, investigated possible caries risk factors in 7–12 year old children in the United States (Leverett *et al.*, 1993a; Leverett *et al.*, 1993b). Here mean baseline fluoride concentrations in saliva of 0.02 – 0.04 ppm were reported in both fluoridated and non-fluoridated drinking water areas with the fluoride concentration being related to caries status rather than drinking water concentration (Leverett *et al.*, 1993a). Studies in the 1990s reported means of about 0.05 ppm fluoride in the saliva of 7–12 year old children regardless of living in fluoridated and non-fluoridated communities (Shields *et al.*, 1995; Shields *et al.*, 1997). Here, after assessment of the caries status of the subjects every 6 months, it was found that

"children with high individual salivary fluoride (0.075 ppm) were more frequently caries free ($p < 0.02$)" (Shields *et al.*, 1995; Shields *et al.*, 1997). Studies have shown that the use of fluoride products (dentifrice, rinse and gels) causes an immediate increase in fluoride concentration in saliva before decreasing with time as fluoride is cleared from the mouth (Bruun *et al.*, 1984; Zero *et al.*, 1992). Fluoride can be retained at concentrations in the saliva between 0.03 and 0.1 ppm for 2–6 hours depending on the product and the individual (Bruun *et al.*, 1984; Zero *et al.*, 1992). Furthermore, it was shown that remineralisation was enhanced in a pH cycling model when exposed to artificial saliva with levels of 0.03 ppm fluoride or higher (Featherstone *et al.*, 1986; Featherstone *et al.*, 1990; Featherstone and Zero, 1992)}. Here a log-linear relationship was revealed between fluoride concentration and degree of protection against caries-like demineralisation; as fluoride concentration increased remineralisation rose with an optimum at approximately 0.08 ppm.

Aims

This study aimed to investigate the effects of continuously delivered low and high levels of fluoride on enamel demineralisation and on members of a biologically relevant oral biofilm community within CDFF caries model systems exposed to sucrose at high frequency. It aimed to examine enamel surfaces as a reservoir of fluoride and further examine the correlation between QLF and TMR in caries quantification.

5.2 Experimental Procedure

The dual model described in Chapter 4 (Bakht *et al.*, 2010; Hope *et al.*, 2012) was used for the basis of this study. It was modified so that one CDFF operated with no added fluoride and the other was exposed to 0.05 ppm fluoride continuously (Figure 5.1). Two, 8-pan CDFFs containing no sample pans were sterilised by dry heat as described

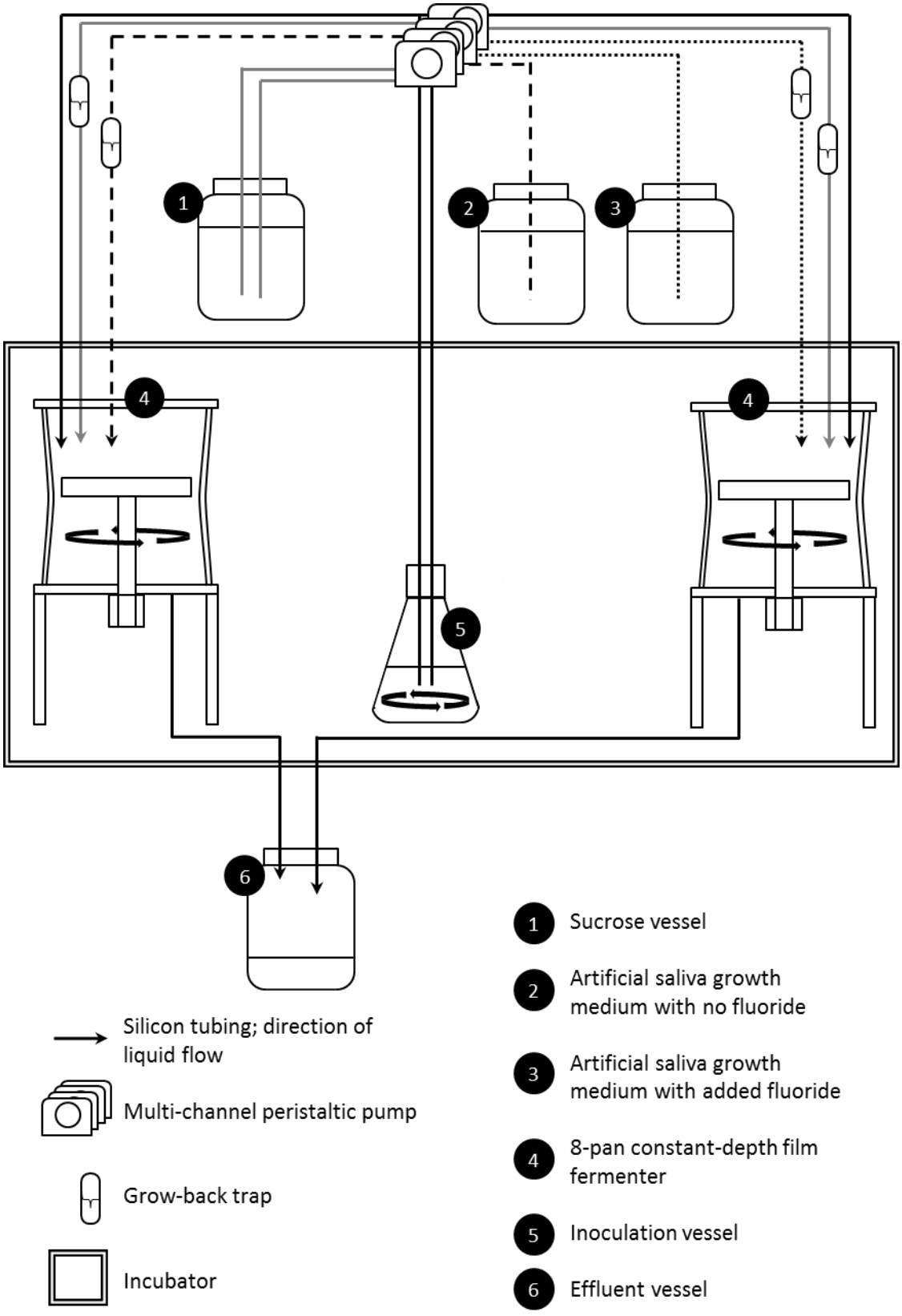


Figure 5.1 Circuit diagram with key of experimental procedure to investigate the effect of 0.05 ppm fluoride on biofilm bacteria and enamel caries within a constant-depth film fermenter model.

and validated in Section 2.5. Ten sample pans were fully loaded with five 4.8 mm HA (Clarkson Chromatography Products, South Williamsport, PA, USA) discs recessed to a depth of 200 μm. Six sample pans were loaded with a combination of 4.8 mm HA (Clarkson Chromatography Products, South Williamsport, PA, USA) and ~5 mm enamel discs (Intertek 4-Front, Chester, UK) recessed to 200 μm also. Of this group, each pan contained three enamel discs, half-painted with nail varnish, and two hydroxyapatite discs (Section 2.4). The sample pans were sterilised by gamma irradiation (Section 2.5) and introduced aseptically into the turntable of each CDFF so that the position of each pan and its contents were comparable between CDFFs. Both CDFFs were inoculated in the same way; connected to the same saliva growth medium inoculated with one aliquot of saliva pool. These methods are fully described in Section 2.5 and in the experimental procedure of Chapter 4 (Section 4.2.). Ten litres of artificial saliva growth media were prepared in one vessel as described in Section 2.5 and thoroughly mixed. The liquid was then split between two vessels and autoclaved. This procedure ensured that the artificial saliva growth media being fed into each side of the dual model was as close to identical as possible. In a laminar flow environment, 2.5 mL artificial saliva growth media was aseptically removed from one vessel and inoculated with 2.5 mL of a sterile 100 ppm fluoride standard (Orion Thermo, Orion Instruments, MA, USA) so that the final concentration of fluoride within this vessel was 0.05 ppm. No fluoride was added to the other vessel. Each vessel was connected aseptically to one of the CDFFs. The contents of each vessel were pumped at 0.267 mL min^{-1} into each CDFF continuously over the course of the experiment as the replenishing media supply. Ten litres of 110 mM sucrose solution (Sigma-Aldrich, Poole, UK) was prepared and autoclaved before being connected to both 8-pan CDFFs. As described in Chapter 3, the sucrose solution was pulsed every 2 hours 8 times daily for 15 mins with a 10 hour rest, at a flow rate of 2.7 mL min^{-1} via a timer controlled peristaltic pump. The final concentration of sucrose, taking into account the dilution effect of the artificial saliva growth media being pumped in at the

same time, was 100 mM. Sucrose flow was first initiated 8 hours after the flow of sterile artificial saliva growth media began. The CDFFs were sampled on day 4, 6, 8, 10, 12 and 14. Sample pans were photographed with biofilm intact using QLF before the microbial composition was elucidated as described in Section 2.7.1.

On day 4, 10 and 14 the sample pans contained three enamel specimens half-painted with nail varnish and were set aside for enamel specimen analysis. The two hydroxyapatite discs provided biofilm for microbial analysis on these days. The enamel discs were photographed with QLF and analysed for fluorescence loss (see Section 2.8.1), scanned for surface changes using NCSP (see Section 2.8.2) and finally subjected to TMR methodology to elucidate any sub-surface changes in mineral content as described in Section 2.8.5. On day 10 and 14 extra sample pans containing biofilm on hydroxyapatite discs only were extracted from each CDFF in the period between sucrose pulses and biofilm pH was determined. The pH of individual biofilm samples contained within each sample pan was obtained in quadruplicate for pans extracted on day 10. The day 14 sample pans were used to investigate biofilm pH changes in response to the addition of sucrose. To measure pH in each instance a micro pH meter was set up so that its probe was in contact with the biofilm but elevated so that it was not in direct contact with the substratum. 20 μL saline, 20 mM or 100 mM sucrose were pipetted onto individual biofilms and the pH was recorded over time.

In a separate CDFF experiment eight sample pans were loaded with a combination of 4.8 mm HA (Clarkson Chromatography Products, South Williamsport, PA, USA) and ~5 mm enamel discs (Intertek 4-Front, Chester, UK). Each pan contained three enamel discs, half-painted with nail varnish, and two hydroxyapatite discs sterilised by gamma irradiation and introduced aseptically into the turntable of the CDFF

(Section 2.5.2). Biofilm was produced as described in Section 2.6 and 110 mM sucrose was pulsed into the CDFF at a flow rate of 5 mL min^{-1} every 2 hours 8 times daily for 15 mins with a 10 hour rest using a timer controlled peristaltic pump. A solution of sodium fluoride (AnalR, BDH, Poole, England, UK) was prepared so that the final concentration was 1450 ppm, then sterilised. From day 4 of the experiment, sodium fluoride solution was delivered continuously into the CDFF at a flow rate of 0.0267 mL min^{-1} using a peristaltic pump and via the side-arm of a grow-back trap in the tubing line feeding sterile artificial saliva growth medium (Figure 5.2). Samples were taken on day 4 before and 1 hour after fluoride flow began, then on days 6, 8, 10, 12 and 14. Selective solid agar was used to isolate and enumerate total viable bacteria, total *Streptococcus* spp, total mutans streptococci group and *Lactobacillus* spp. Enamel discs half painted with nail varnish were photographed with QLF and analysed for fluorescence loss (see Section 2.8.1), scanned for surface changes using NCSP (see Section 2.8.2) and finally subjected to TMR methodology to elucidate any sub-surface changes in mineral content as described in section 2.8.5. Enamel samples from day 4 and 14 were also studied using SEM/EDX (Section 2.8.3).

5.2.1. Statistical Analyses

The hypothesis that there will be differences in enamel mineral content (as assessed by QLF and TMR) and in surface changes (quantified by NCSP) when exposed to microcosm biofilm subjected to caries challenge alone or together with 0.05 ppm or 1,450 ppm fluoride continuously was tested using single factor ANOVA and two-way ANOVA analyses. Sidak's multiple comparisons tests were used post-hoc to identify the statistical significance of differences between outcomes (a) at each time within a given experiment and (b) for differences between each experiment for each time point. An analysis of covariance (ANCOVA) model able to adjust for time was used to test the hypothesis that 0.05 ppm fluoride affects the growth of microbial constituents of the biofilm. Pearson's product moment coefficients (r) were calculated to determine

how well QLF and TMR correlate in the assessment of caries lesions formed in the presence or absence of fluoride. One-way ANOVA tested for statistical significance in differences in pH of biofilm exposed to 0.05 ppm fluoride continuously or not. Prior to all analysis normal distributions of the raw data was checked and transformed where needed and all datasets were routinely checked for outliers as described in Section 2.9.

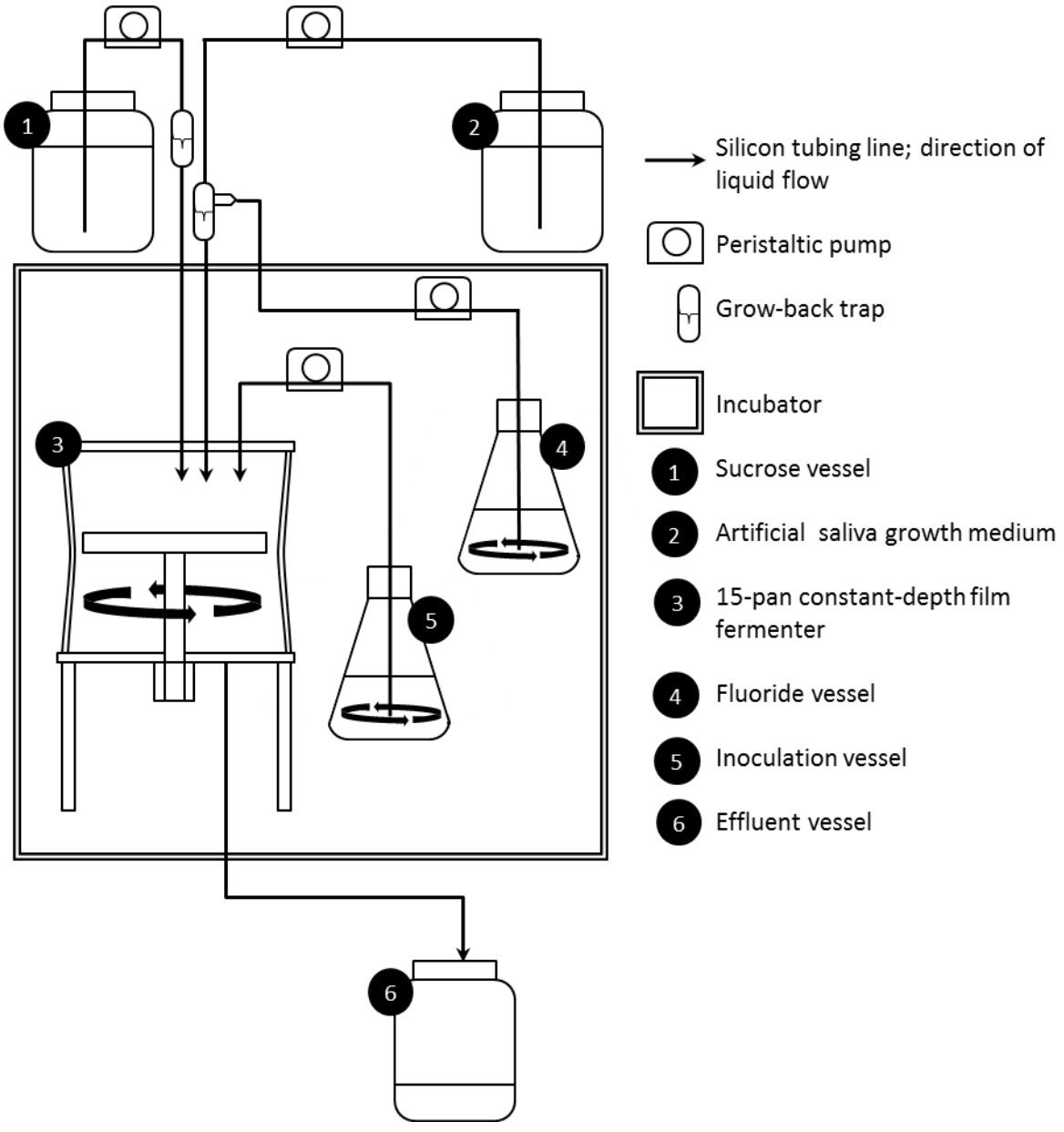


Figure 5.2. Circuit diagram with key of experimental procedure to investigate the effect of continuous low flow exposure to 1,450 ppm fluoride on caries progression within a biologically relevant constant-depth film fermenter model of caries.

5.3 RESULTS

5.3.1 Effect of continuous 0.05 ppm fluoride exposure on enamel demineralisation

Both QLF and TMR distinguished higher degrees of caries in enamel beneath biofilm exposed to 0.05 ppm fluoride continuously within the artificial saliva growth medium supply and 100 mM sucrose pulsing than in enamel exposed to the caries challenge alone (Figures 5.3 – 5.5 and Table 5.1). By day 14 fluorescence change (ΔF, %) was -67.30 ± 1.50 SD (n = 3) in 0.05 ppm fluoride exposed samples compared to -46.701 ± 7.30 SD (n = 3) in samples with no additional fluoride exposure. Mineral loss (ΔZ, %Vol.μm) and lesion depth (μm) were 2964.06 ± 193.13 SD (n = 3) and 94.75 ± 1.71 SD (n = 3) respectively in fluoride exposed enamel whilst ΔZ and lesion depth were 2517.50 ± 441.06 SD (n = 3) and 86.98 ± 7.76 SD (n = 3) in enamel exposed to the caries challenge alone. Statistical analysis of ΔF revealed that differences were statistically significant according to fluoride exposure (p = 0.0095), time (p < 0.0001) and the interaction of the two factors (p = 0.0154). Subsequent multiple comparisons testing showed that when comparing between fluoride exposed and non-fluoride exposed groups differences in ΔF were statistically significant only at day 14 (p = 0.0004; Figure 5.3a). Differences in both mineral loss and lesion depth were only statistically significant according to the factor of time (p = 0.0009 and p = 0.0004 respectively); accounting for 90.9% and 93.2% of the total variation respectively.

Table 5.1. Lesion depth (μm) determined by TMR in bovine enamel samples exposed to multispecies oral biofilm pulsed with 100 mM sucrose 8 times daily and either no fluoride or 0.05 ppm fluoride continuously. Data is for mean ± standard deviation, n = 3

Exp. conditions	Lesion depth (μm) over time (day)		
	4	10	14
100 mM sucrose alone	24.44 ± 2.47	54.82 ± 18.19	86.98 ± 7.76
100 mM sucrose + 0.05 ppm F^-	21.73 ± 2.98	65.66 ± 9.05	94.75 ± 1.71

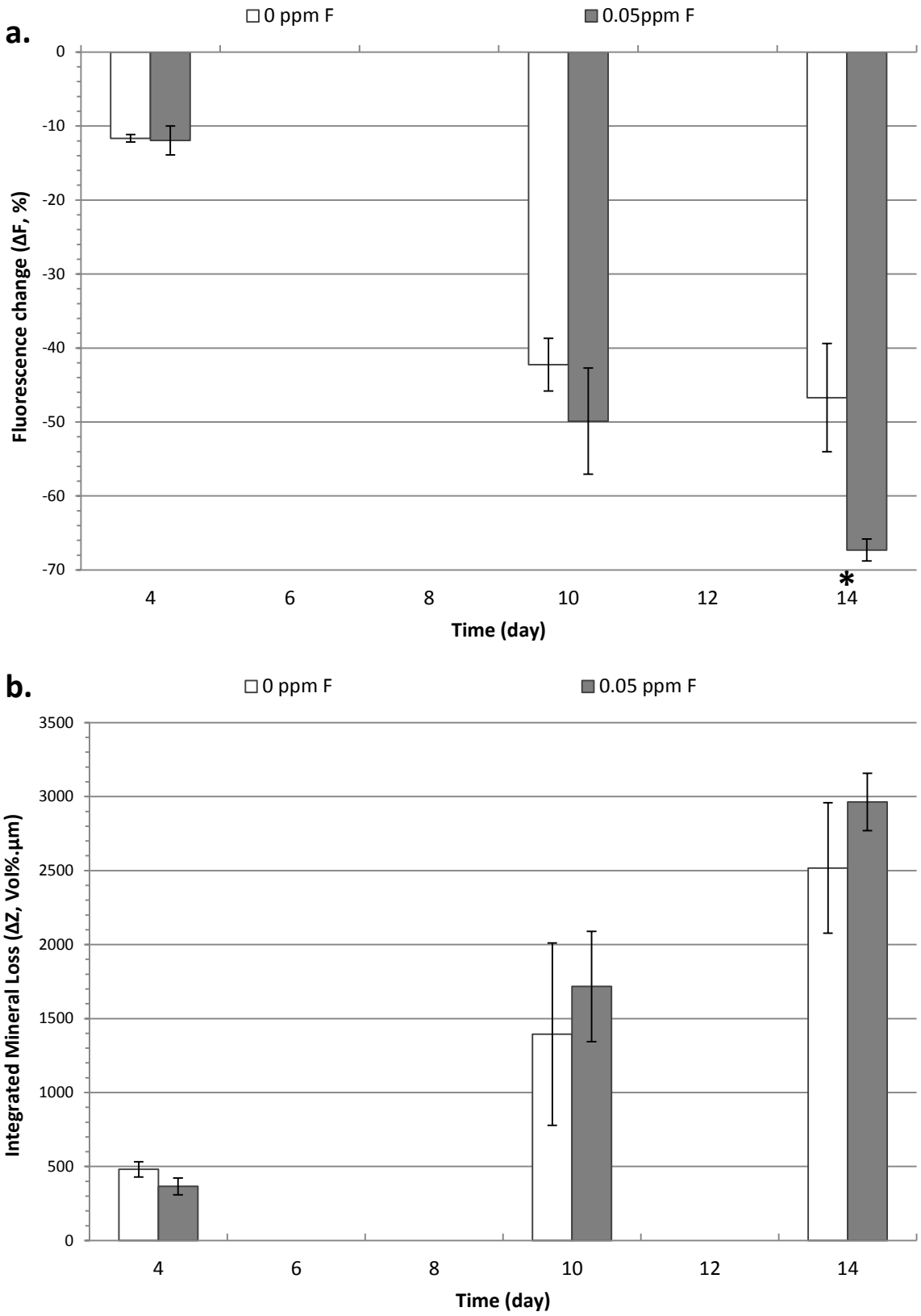


Figure 5.3. Longitudinal changes in (a) enamel fluorescence (average fluorescence loss, ΔF, %) as detected by QLF *in vitro* and (b) mineral loss (average ΔZ, %Vol.µm) determined by TMR in bovine enamel samples exposed to multispecies oral biofilm pulsed with 100 mM sucrose 8 times daily and either no fluoride (white bars) or 0.05 ppm fluoride (grey bars) continuously. Error bars represent standard deviation, $n = 3$. ***** indicates $p < 0.01$.

Figure 5.4. Typical QLF-D images of bovine enamel sampled longitudinally within a biological caries model subjected to either no fluoride or 0.05 ppm fluoride continuously. Right side of enamel sample was exposed to experimental conditions; left was protected as internal control.

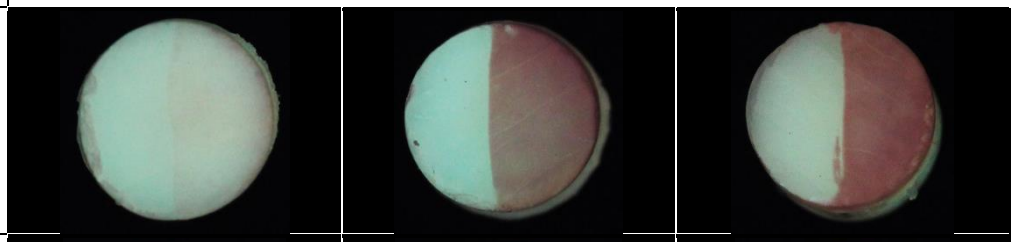
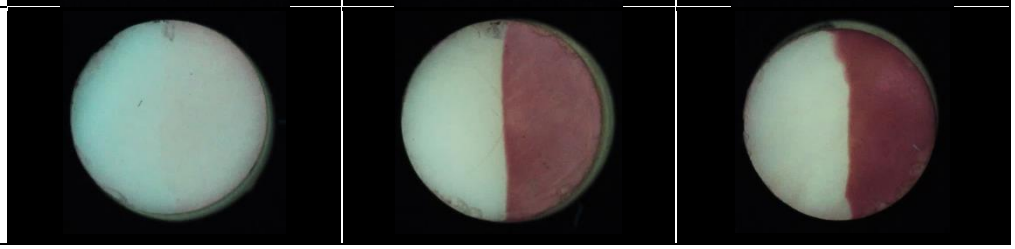
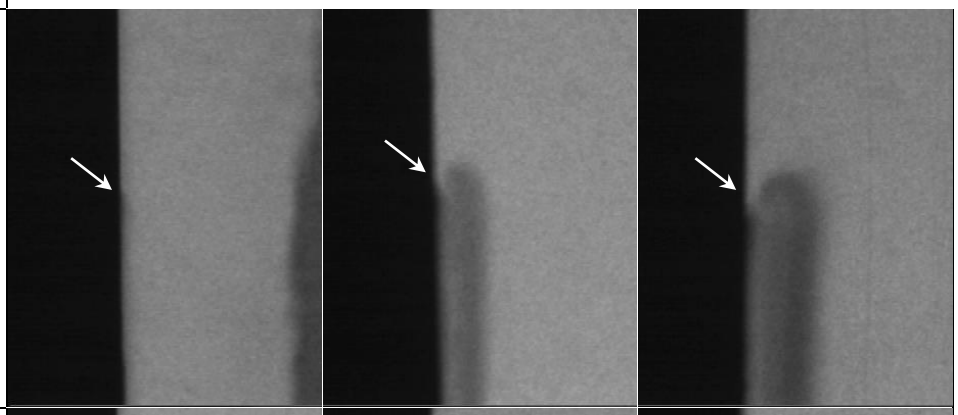
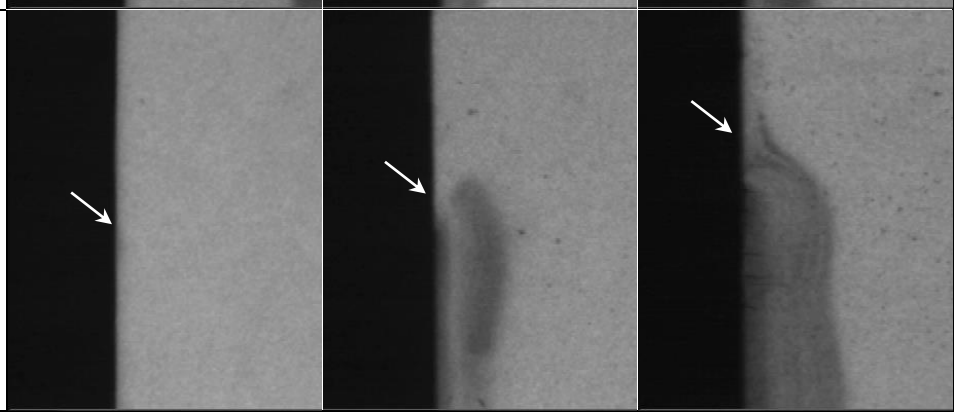
Exp. conditions	Time (day)		
	4	10	14
100 mM sucrose alone			
100 mM sucrose + 0.05 ppm F^-			

Figure 5.5. Typical TMR images of bovine enamel sampled longitudinally within a biological caries model subjected to either no fluoride or 0.05 ppm fluoride continuously. Arrow indicates the interface between the biofilm exposed and nail-varnish protected zones.

Experimental conditions	Time (day)		
	4	10	14
100 mM sucrose alone			
100 mM sucrose + 0.05 ppm F^-			

Sidak's multiple comparisons test comparing between experimental conditions with regards to mineral loss and lesion depth (Figure 5.3b and Table 5.1) revealed no statistically significant differences throughout the experiment (p = 0.985/0.942 at day 4, p = 0.783/0.620 at day 10 and p=0.587/0.808 at day 14 for mineral loss/lesion depth respectively). Meanwhile, in enamel exposed to the caries challenge alone, differences between day 4 samples and day 14 samples was statistically significant as determined by ΔF, ΔZ and lesion depth (Table 5.2). Differences between day 4 and day 10 samples were significant only in terms of fluorescence changes and lesion depth whilst lesion depth was the only parameter elucidating day 10 and 14 samples as significantly different. Differences in enamel exposed 0.05 ppm fluoride in addition to the caries challenge for 4 or 14 days were also statistically significant in terms of ΔF, ΔZ and lesion depth and when comparing day 4 and day 10 samples according all three descriptors of enamel demineralisation (Table 5.3). When comparing day 14 samples with those of day 10 QLF did not distinguish a statistically significant difference while TMR did according to both ΔZ and lesion depth (Table 5.3).

Table 5.2. Results of multiple comparisons testing the statistical significance between changes in enamel caries characteristics over 14 days in a caries model exposed to **100 mM** sucrose 8 times daily according to fluorescence change, mineral loss, lesion depth and surface loss (n = 3 throughout).

Time (day)	Fluorescence change (ΔF, %)			Mineral Loss *(ΔZ, %Vol.μm)*			Lesion Depth (μm)			Surface Loss* (μm)		
	MD	±CI$^{95\%}$	p	MD	±CI$^{95\%}$	p	MD	±CI$^{95\%}$	p	MD	±CI$^{95\%}$	p
4 vs 10	30.6	19.2, 42.0	**0.0001**	-914	-2042, 215	0.0964	-30.4	-57.3, -3.49	**0.0333**	-0.007	-0.034, 0.020	0.8747
4 vs 14	35.0	23.6, 46.4	**< 0.001**	-2036	-3165, -908	**0.0062**	-62.5	-89.4, -35.6	**0.0024**	0.0003	-0.026, 0.027	> 0.999
10 vs 14	4.46	-6.94, 15.9	0.6163	-1122	-2251, 6.14	0.0509	-32.2	-59.0, -5.26	**0.0274**	0.0071	-0.020, 0.0338	0.8587

Mean difference, MD; upper and lower confidence intervals, ±CI$^{95\%}$

*$\log_{10}$ transformed prior to analysis; **bold** font denotes difference is statistically significant

Table 5.3. Results of multiple comparisons testing the statistical significance between changes in enamel caries characteristics over 14 days in a caries model exposed to 100 mM sucrose 8 times daily and **0.05 ppm fluoride** continuously according to fluorescence change, mineral loss, lesion depth and surface loss (n = 3 throughout).

Time (day)	Fluorescence change (ΔF, %)			Mineral Loss *(ΔZ, %Vol.μm)*			Lesion Depth (μm)			Surface Loss* (μm)		
	MD	±CI$^{95\%}$	p	MD	±CI$^{95\%}$	p	MD	±CI$^{95\%}$	p	MD	±CI$^{95\%}$	p
4 vs 10	37.9	26.5, 49.3	**< 0.001**	-1351	-2480, -223	**0.0273**	-46.0	-72.9, -19.2	**0.0075**	-0.011	-0.038, 0.015	0.598
4 vs 14	55.4	44.0, 66.8	**< 0.001**	-2598	-3727, -1470	**0.0025**	-75.1	-102, -48.2	**0.0012**	-0.017	-0.044, 0.009	0.264
10 vs 14	17.4	6.02, 28.8	0.053	-1247	-2376, -118	**0.0359**	-29.1	-56.0, -2.20	**0.0386**	-0.006	-0.033, 0.021	0.906

Mean difference, MD; upper and lower confidence intervals, ±CI$^{95\%}$

*$\log_{10}$ transformed prior to analysis; **bold** font denotes difference is statistically significant

TMR lesion profiles compiled as averages from all sections obtained in all enamel discs sampled over the course of the experiment confirmed the progression of enamel demineralisation over time in samples exposed to no fluoride or 0.05 ppm fluoride continuously (Figure 5.6a & Figure 5.6b respectively). These figures confirm no subsurface demineralisation at day 4 in both groups and that subsurface demineralisation is present in both groups by day 10, though to a larger extent with deeper clearly formed lesions in 0.05 ppm fluoride exposed enamel. The shape of lesion profiles at day 10 and 14 in particular, indicate that a degree of surface softening also occurred and this is confirmed in the images of Figure 5.5. By day 14, in lesions within enamel exposed to the caries challenge alone a subsurface demineralisation zone could be clearly distinguished regardless of surface softening while in those exposed to added fluoride the demineralisation zone was broad with less mineral intact at the enamel surface.

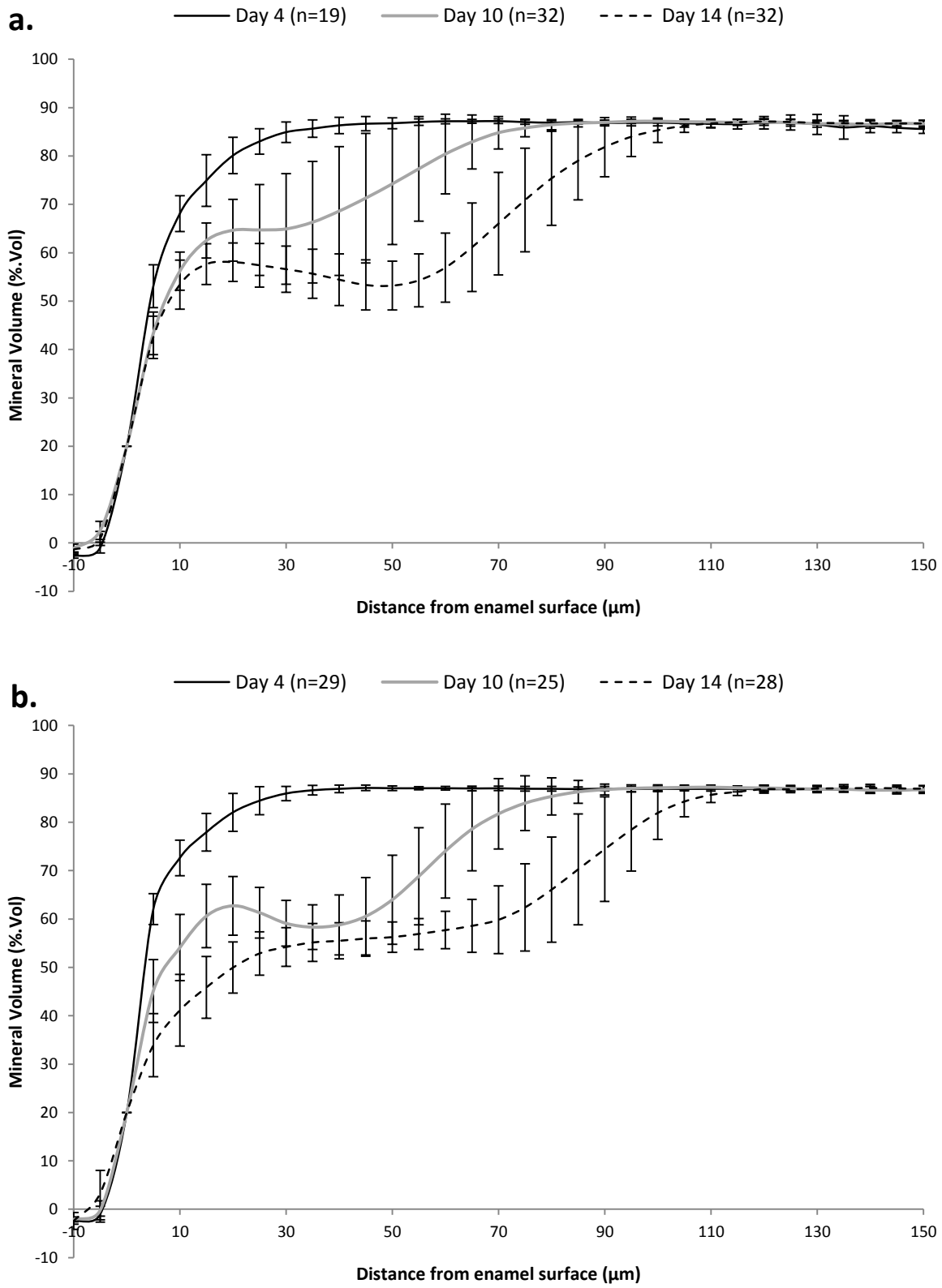


Figure 5.6. TMR lesion profiles indicating mineral volume (%.Vol) for caries in bovine enamel samples exposed to multispecies oral biofilm pulsed with 100 mM sucrose 8 times daily and either (a) no fluoride or (b) 0.05 ppm fluoride continuously. Averages from all sections obtained in enamel exposed for 4, 10 or 14 days are compared. Error bars represent standard deviation, *n* shown in parentheses beside each group in figure legend.

However, TMR revealed the presence of lamination zones within the lesions of samples exposed to 0.05 ppm fluoride for 14 days, none in samples exposed for 4 days and the presence of a combination of laminated and non-laminated regions in day 10 samples (Table 5.8 and Figure 5.9). Interesting variation could be seen in enamel demineralisation along the length of the exposed side of each disc. Table 5.8 summarises images taken equidistant along the lesion length in the direction away from the interface between the biofilm exposed and nail-varnish protected areas of the enamel disc (illustrated in Figure 5.7). Laminations were present throughout the length of lesions in enamel exposed for 14 days as demonstrated by the example included (Table 5.8d and Figure 5.9d) and were present in all 4 sections taken across all 3 enamel discs of this group. In one enamel disc exposed to 0.05 ppm fluoride for 10 days all sections possessed lamination zones closest to the interface between the exposed and non-exposed sides of the disc before becoming non-laminated further towards the edge of the section (Table 5.8a and Figure 5.9a). In another disc of this group, single lesion bodies with no laminations were present in 3 of the sections (Table 5.8b and Figure 5.9b) yet in one section (Table 5.8c and Figure 5.9c) laminations were present closest to the interface before disappearing into non-laminated lesion bodies as the edge of the section was approached.

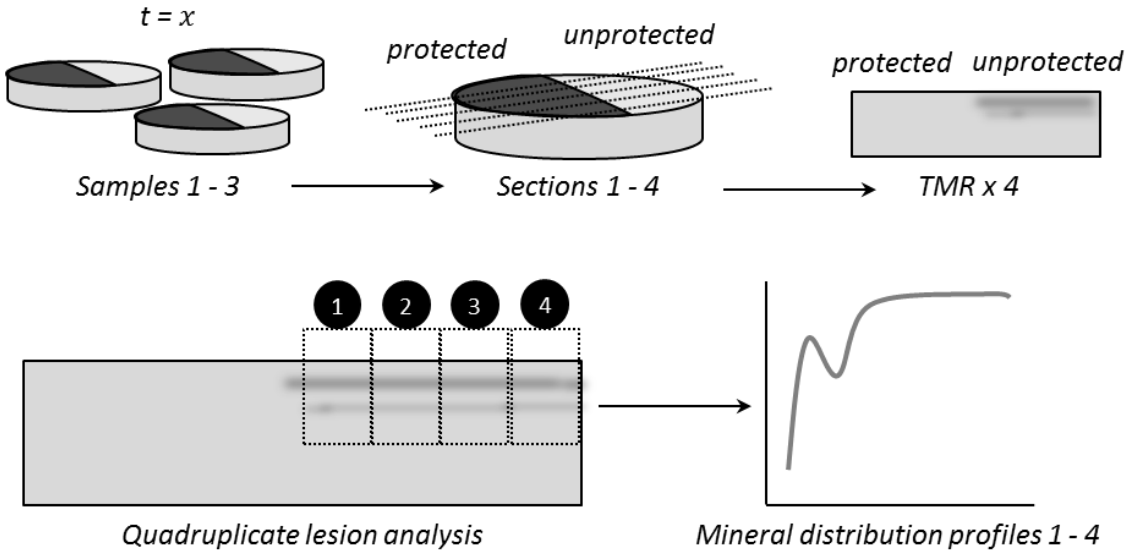
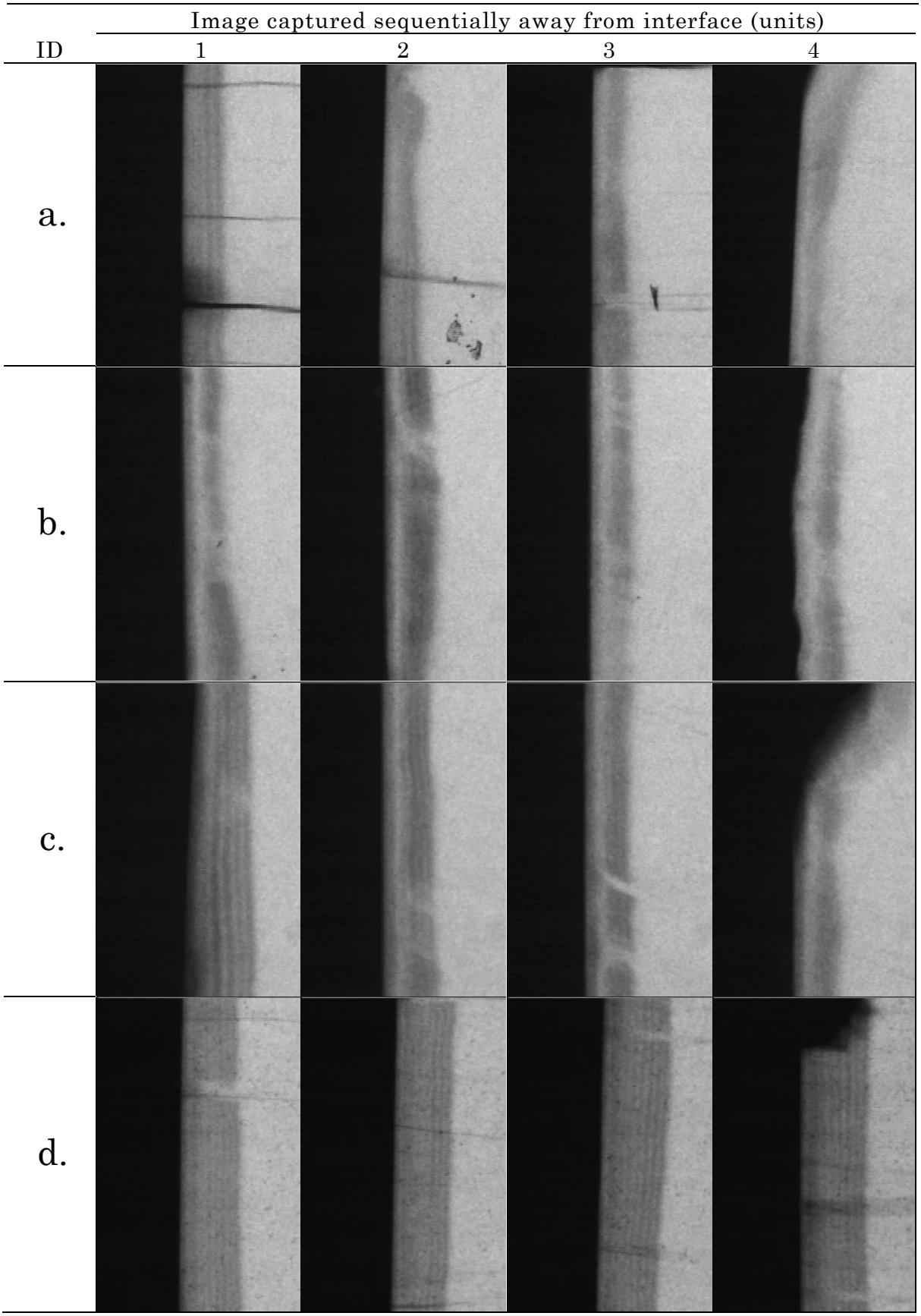


Figure 5.7. Technique employed when capturing a representative cross-section of mineral profiles within caries lesions using TMR.

Figure 5.8 Example TMRs of caries lesions in enamel beneath cariogenic biofilm pulsed with 100 mM sucrose and 0.05 ppm fluoride. (a) section representative of **day 10 sample 1** (b) **day 10 sample 2**, no laminations (c) another section from **day 10 sample 2** (d) typical of all **day 14** sections.



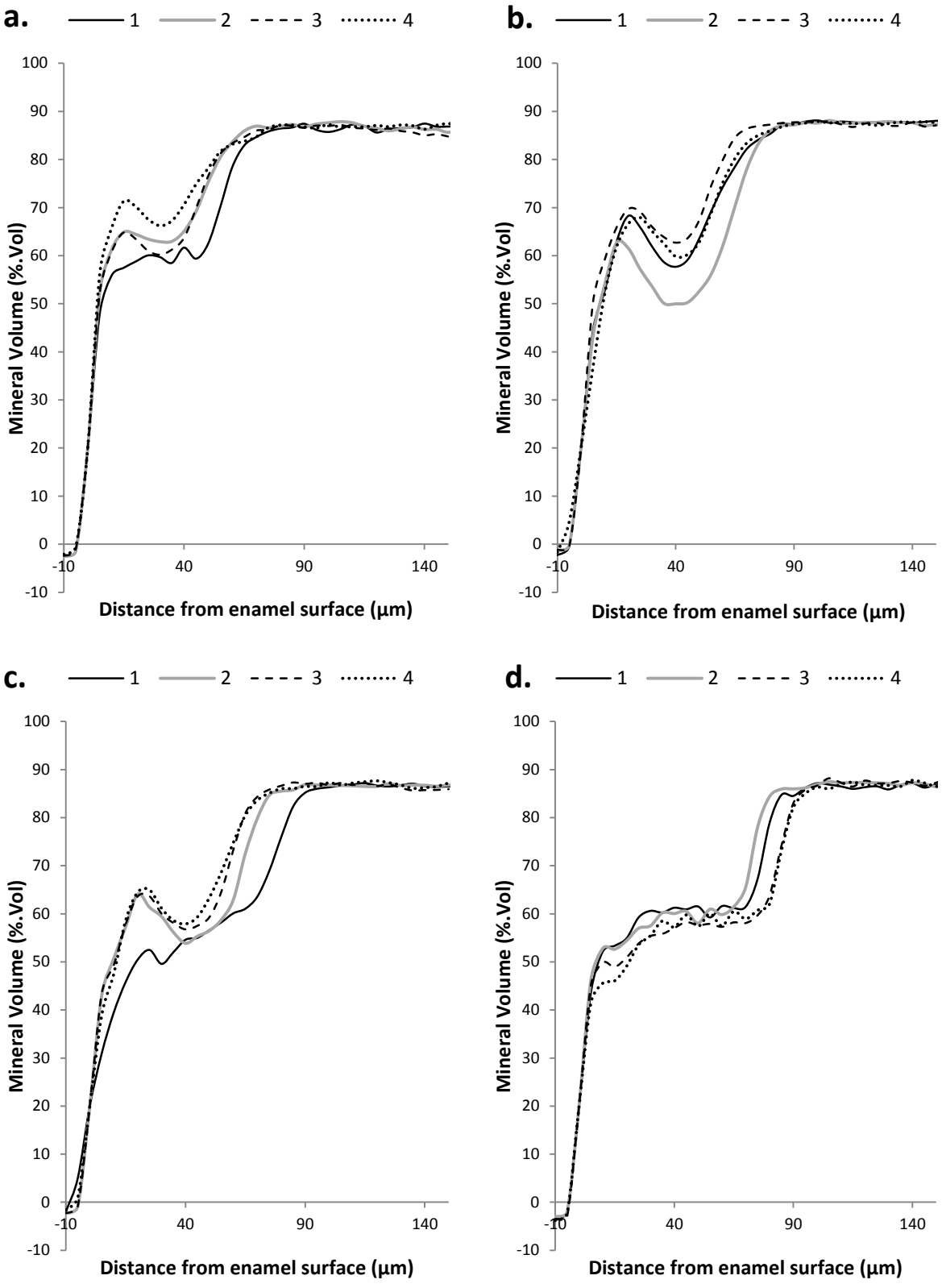


Figure 5.9. TMR data profiles of caries lesions presented in **table 5.6**. (a) **day 10** sample 1, section 1; (b) **day 10** sample 2, section 1; (c) **day 10** sample 2, section 2; (d) **day 14** sample 1, section 1. Numbers in graph legends denote profile for each image captured equidistant along the length of each section in the direction away from the interface between the biofilm exposed and nail-varnish protected zones.

5.3.2 Relationship between QLF and TMR in quantifying caries lesions with lamination zones

QLF and TMR appear to detect trends in enamel demineralisation similarly (Figure 5.3) and TMR revealed the presence of lamination zones within the lesions of 0.05 ppm fluoride exposed enamel. Subsequently, the correlation between TMR ΔZ and QLF ΔF when laminated zones are present was inspected. When comparing between these variables paired for all individual replicate samples correlation was strong (r = 0.94, p = 0.006, n = 6; Figure 5.10) and the existence of a straight-line relationship between variables was evident (p = 0.006, df = 1, F = 29.498). The adjusted r^2 concluded that 85.1% of ΔF could be explained by ΔZ or vice-versa. Furthermore, it was shown that no autocorrelation was present in this dataset (d = 2.144, n = 6, k = 2) providing evidence that observations were independent.

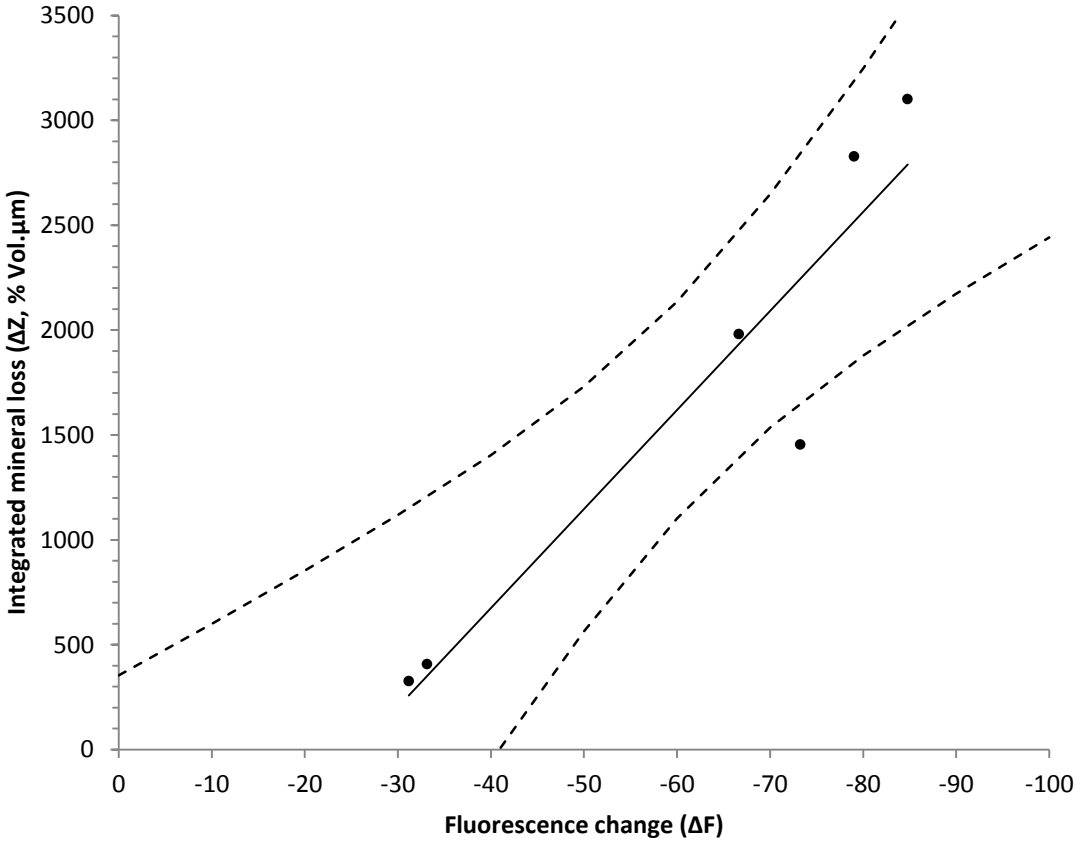


Figure 5.10. Scatter plot of the correlation between fluorescence change assessed by QLF and integrated mineral loss measured using TMR in a biologically relevant caries model of snacking consumption pulsed continuously with 0.05 ppm fluoride. Dashed lines represent upper and lower 95% confidence bounds.

5.3.3 Effect of caries challenge and 0.05 ppm fluoride on enamel surface loss

NCSP revealed longitudinal trends of surface loss as similar to those observed by the QLF and TMR quantification of enamel demineralisation (Figure 5.11). Less surface loss was observed in samples exposed to 100 mM sucrose 8 times daily alone than in those exposed to 0.05 ppm fluoride continuously in addition to the same caries challenge by day 10 (0.021 ± 0.031 SD µm, n = 3 vs 0.025 ± 0.025 SD µm, n = 3 respectively) and in day 14 (0.004 ± 0.018 SD µm, n = 3 vs 0.040 ± 0.054 SD µm, n = 3 respectively). However, differences were not significantly different over time (p = 0.362) or whether fluoride was present or not (p = 0.443). At 4 days of exposure, surface loss was quantified in enamel exposed to the caries challenge alone (0.005 ± 0.005 SD µm, n = 3) compared to no surface loss in enamel exposed to fluoride in addition (-0.002 ± 0.004 SD µm, n = 3).

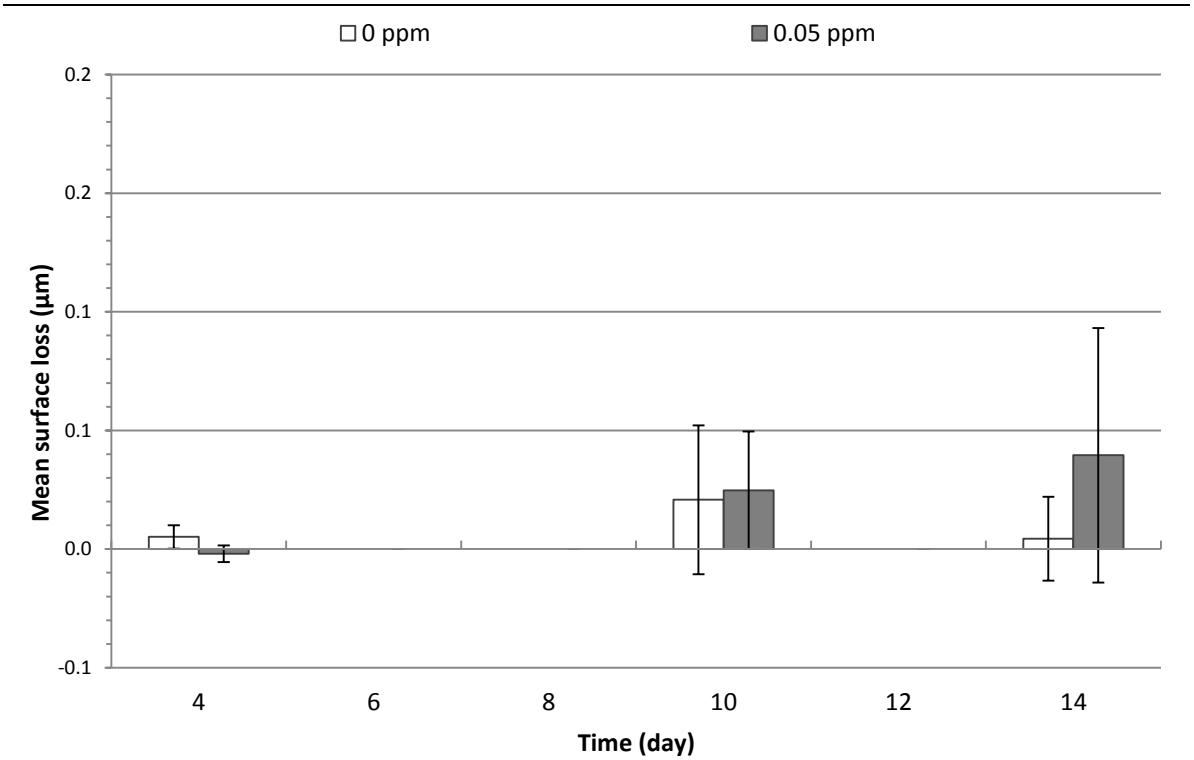


Figure 5.11. Mean surface loss (µm) of bovine enamel samples exposed to multispecies oral biofilm pulsed with 100 mM sucrose 8 times daily and either no fluoride or 0.05 ppm fluoride continuously as detected by NCSP measurements of height differences between negative control and treatment zones within samples. Error bars represent standard deviation, n = 3.

5.3.4 Biofilm formation and growth in the continuous presence of 0.05 ppm fluoride

Figure 5.12 shows the $\log_{10}$ transformed microbial composition of multispecies oral biofilm when exposed to (a) no fluoride or to (b) 0.05 ppm fluoride throughout biofilm formation and development with additional pulses of 100 mM sucrose 8 times daily in both. Counts of $\log_{10}$ total viable bacteria showed a pseudo-steady state was achieved in all biofilm by day 4. Results indicate that *Lactobacillus* spp. growth was supressed in biofilm exposed to 0.05 ppm fluoride; only present in detectable levels at day 10 (2.76 ± 0.98 SD cfu mm^{-2}, $n = 4$) and at no other day. Conversely, when no added fluoride was present *Lactobacillus* spp. growth reached $9.08 \times 10^6 \pm 9.77 \times 10^4$ SD cfu mm^{-2} ($n = 4$) by day 10 and $2.57 \times 10^7 \pm 6.24 \times 10^6$ SD cfu mm^{-2} ($n = 4$) by day 14. Mutans streptococci of day 4 biofilm exposed to 0.05 ppm fluoride were present at significantly lower levels than when exposed to no fluoride; reaching $6.29 \times 10^1 \pm 1.61 \times 10^1$ SD cfu mm^{-2} ($n = 4$) compared to $3.59 \times 10^4 \pm 2.65 \times 10^4$ SD cfu mm^{-2} ($n = 4$) respectively. Subsequently, counts of mutans streptococci rose in both treatments at day 6 but numbers were again significantly lower in fluoride exposed than in non-fluoride exposed biofilm ($3.16 \times 10^4 \pm 1.95 \times 10^3$ SD cfu mm^{-2} versus $9.43 \times 10^5 \pm 8.21 \times 10^5$ SD cfu mm^{-2} respectively, $n = 4$). Mutans streptococci appear to have achieved pseudo-steady state by day 8 in the presence of no fluoride or 0.05 ppm F^-; $1.93 \times 10^4 \pm 9.58 \times 10^3$ SD cfu mm^{-2} ($n = 4$) and $1.16 \times 10^4 \pm 1.22 \times 10^4$ SD cfu mm^{-2} ($n = 4$) respectively (average of day 8 – 14 counts, $n = 4$). At day 14 numbers of mutans streptococci appear to be higher in 0.05 ppm fluoride exposed biofilm ($2.80 \times 10^4 \pm 8.79 \times 10^3$ SD cfu mm^{-2}, $n = 4$) than in biofilm exposed to no additional fluoride ($1.73 \times 10^4 \pm 2.65 \times 10^4$ SD cfu mm^{-2}, $n = 4$). Differences in total bacteria and total *Streptococcus* spp., and mutans streptococci were statistically significant when comparing bacterial counts of non-fluoride and fluoride exposed biofilm (Tables 5.4 – 5.6) yet differences in viable counts of *Lactobacillus* spp. were not ($p = 0.095$; Table 5.7). The effect of time was statistically significant for *Streptococcus* spp. and *Lactobacillus* spp. only while

the interaction between fluoride exposure and time was statistically significant in mutans streptococci and *Lactobacillus* spp. Statistically significant interactions suggest that mutans streptococci and *Lactobacillus* spp. counts changed in markedly different ways at each time point when exposed to fluoride or not.

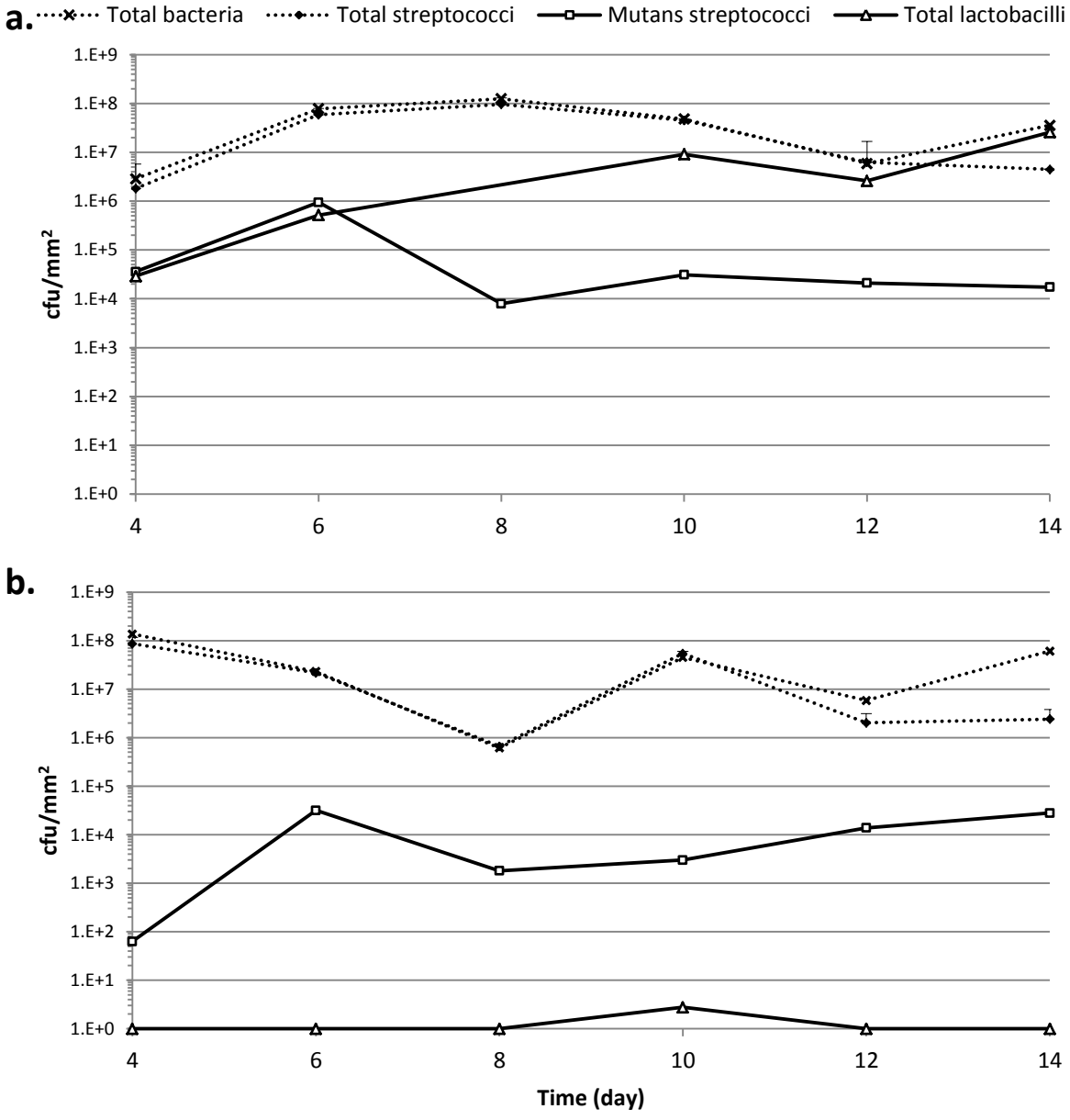


Figure 5.12. Microbial composition of multispecies oral biofilm grown on hydroxyapatite exposed to (a) no fluoride or (b) 0.05 ppm fluoride artificial saliva growth media. Both pulsed with 100 mM sucrose eight times daily. Error bars represent standard deviation, $n = 4$ (technical replicates).

Table 5.4. Results of ANCOVA testing statistical significance of time and fluoride addition in **total viable bacteria*** exposed to 100 mM sucrose and either **no or 0.05 ppm fluoride continuously**.

Source of Variation	SSIII	*df*	MS	F	*p*
Fluoride	342.612	2	643.466	312.843	**< 0.001**
Time	0.000	1	0.000	0.000	0.983
Fluoride x time	0.332	1	0.332	0.641	0.428

R Squared = 0.991 (Adjusted R Squared = 0.990)

Table 5.5. Results of ANCOVA testing statistical significance of time and fluoride addition in **total *Streptococcus* spp.*** exposed to 100 mM sucrose and either **no or 0.05 ppm fluoride continuously**.

Source of Variation	SSIII	*df*	MS	F	*p*
Fluoride	359.382	2	179.691	1262.419	**< 0.001**
Time	3.091	1	3.091	6.556	**0.014**
Fluoride x time	1.681	1	1.681	3.565	0.066

R Squared = 0.991 (Adjusted R Squared = 0.991)

Table 5.6. Results of ANCOVA testing statistical significance of time and fluoride addition in **mutans streptococci*** exposed to 100 mM sucrose and either **no or 0.05 ppm fluoride continuously**.

Source of Variation	SSIII	*df*	MS	F	*p*
Fluoride	111.997	2	55.998	19.597	**< 0.001**
Time	0.960	1	0.960	0.336	0.565
Fluoride x time	15.465	1	15.465	5.412	**0.025**

R Squared = 0.825 (Adjusted R Squared = 0.809)

Table 5.7. Results of ANCOVA testing statistical significance of time and fluoride addition in **total *Lactobacillus* spp.*** exposed to 100 mM sucrose and either **no or 0.05 ppm fluoride continuously**.

Source of Variation	SSIII	*df*	MS	F	*p*
Fluoride	13.338	2	6.669	2.489	0.095
Time	16.799	1	16.799	6.269	**0.016**
Fluoride x time	15.507	1	15.507	5.787	**0.020**

R Squared = 0.851 (Adjusted R Squared = 0.837)

Type III sum of squares, SSIII; degrees of freedom, *df*; mean square, MS; F = (MS between groups)/(MS within groups). *$\log_{10}$ transformed prior to analysis. **bold** font denotes difference is statistically significant.

However, when time as a covariate is discounted from the statistical model, estimated marginal means analysis revealed the statistical significance of differences in bacterial counts in response to the effect of fluoride alone (Table 5.8). Differences in *Lactobacillus* spp. and mutans streptococci were statistically significant when fluoride was added. Whereas statistical significance was not found for differences in counts of *Streptococcus* spp. and total bacteria in response to fluoride addition when time was accounted for.

5.3.5 pH observations and pH response to sucrose addition in biofilm exposed to 0.05 ppm fluoride continuously

For samples extracted on day 10, pH was obtained at four distinct points across three biofilm samples in each pan. Here biofilm pH was fairly acidic for both groups but was, on average, 0.109 units lower in 0.05 ppm fluoride exposed biofilm compared to biofilm exposed to the 100 mM sucrose caries challenge alone (pH 5.505 ± 0.112 vs 5.614 ± 0.211 respectively, $n = 12$ for each). This difference was found not to be statistically significant following one-way ANOVA testing ($p = 0.132$, F (1, 22) = 2.453) and Levene's test observed homogeneity of variances (5.431, $p = 0.029$). Figure 5.13 illustrates the diversity of pH observed in replicate readings of individual biofilm samples and that in different parts of the biofilm pH was below critical pH (5.5) for both test groups. The pH response to the addition of 100 mM sucrose measured in

Table 5.8. Results of statistical testing examining the effect of 0.05 ppm fluoride addition on constituents of multispecies oral biofilm based on linearly independent pairwise comparisons among estimated marginal means.

	Bacterial group			
Fluoride exposure comparisons	Total bacteria*	*Streptococcus* spp.*	Mutans streptococci*	*Lactobacillus* spp.*
0.05 ppm fluoride vs no fluoride	F = (1,44) 0.567 $p = 0.455$	F = (1,44) 1.694 $p = 0.200$	F = (1,44) 4.676 **$p = 0.036$**	F = (1,44) 115.484 **$p < 0.001$**

F-statistic; F. *$\log_{10}$ transformed prior to analysis.

bold font emphasises difference is statistically significant.

replicate for biofilm exposed to 0.05 ppm fluoride continuously alongside the caries challenge regime for 14 days (Figure 5.14) showed the same overall trend. Though no Stephan-like pH curve was detected, under these conditions, baseline pH was as low as pH 5.5 and the final pH after 2 minutes was pH 5.42.

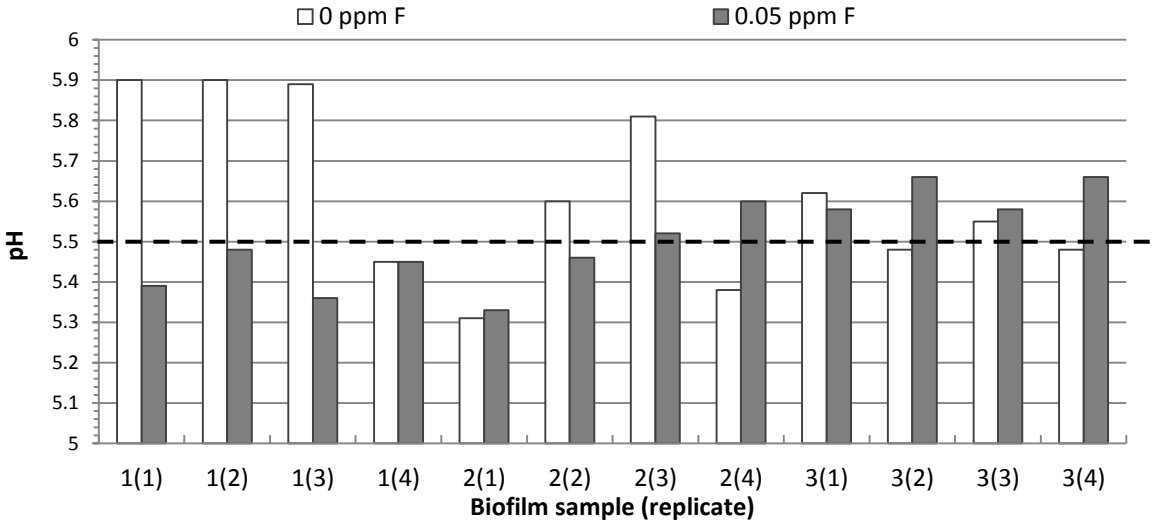


Figure 5.13. pH of biofilm exposed to 100 mM sucrose 8 times daily and either no fluoride (white bars) or 0.05 ppm fluoride continuously (grey bars). Quadruplicate pH readings (1-4) for three biofilm samples contained within one CDFF sample pan in each test group are presented. Dashed horizontal line indicates critical pH.

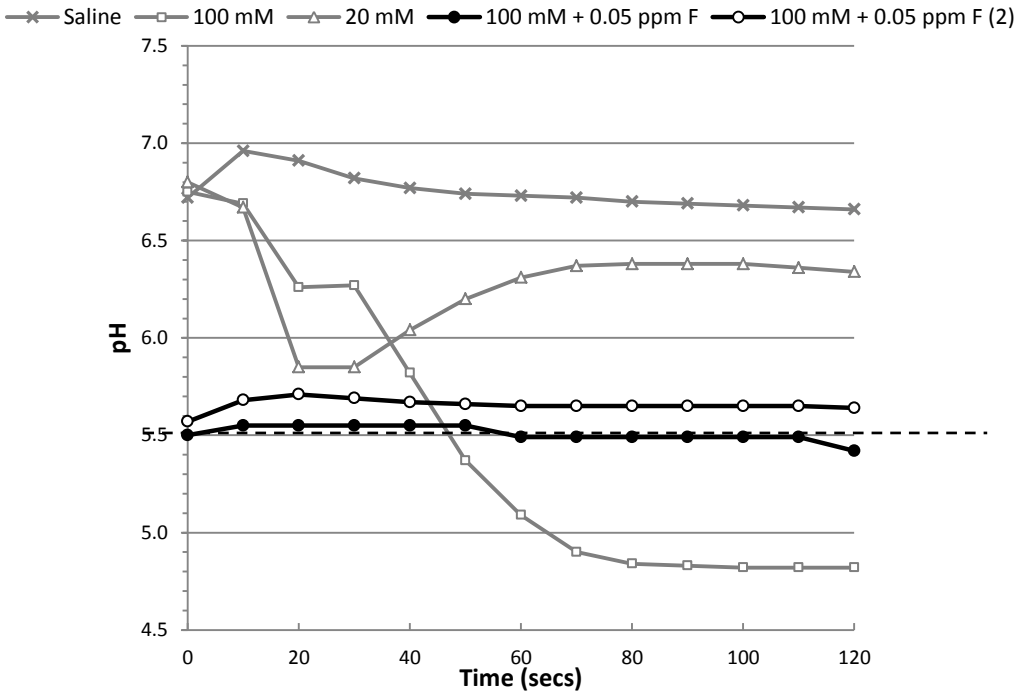
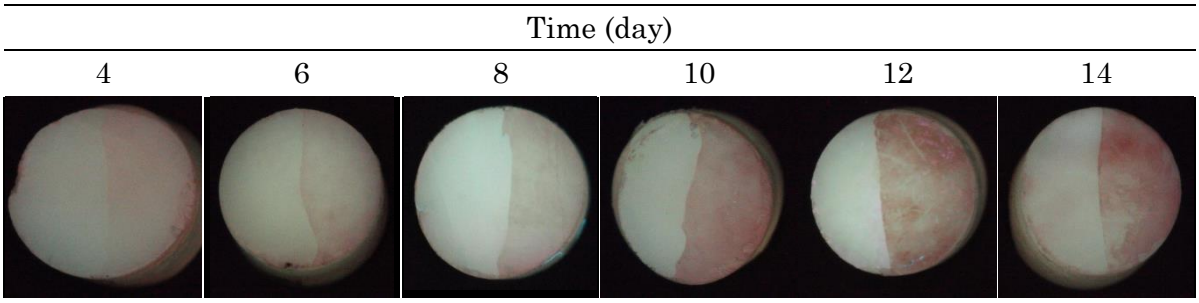


Figure 5.14. Biofilm pH response curves to the addition of saline, 100 mM sucrose or 20 mM sucrose. Black line profiles represent pH changes in response to the addition of 100 mM sucrose for biofilm grown under continuous exposure to 0.05 ppm fluoride and 100 mM sucrose 8 times daily. Grey line profiles are data from a previous study (Section 3.3.6) for reference. Dashed horizontal line indicates critical pH.

5.3.6 Effect of continuous 1,450 ppm fluoride exposure on enamel demineralisation and surface loss

Both QLF and TMR quantified some demineralisation in enamel beneath biofilm exposed to 100 mM sucrose eight times daily and an additional continuous supply of 1,450 ppm sodium fluoride (Figures 5.15 – 5.17). Figure 5.17 summarises microradiograph images taken equidistant along the length of the enamel in the direction away from the interface between the biofilm exposed and nail-varnish protected areas of the enamel disc (as illustrated in Figure 5.7). TMR revealed arrested surface erosion lesions (e.g. Figure 5.17; day 12) and small levels of surface softening (e.g. Figure 5.17; day 4, 6 and 14) that extended less than 10 and 20 µm, respectively, into the enamel structure of these samples and no subsurface demineralisation. If frank erosive lesions were present they appear most prominent at the junction between these areas. Subsurface lesions characteristic of caries were observed in three sections of one disc sampled at day 10 (e.g. Figure 5.17; day 10) with a lesion depth of 23.725 ± 1.23 µm (n = 4). The fourth section derived from this disc exhibited minute surface erosion but no subsurface demineralisation. The largest fluorescence change (ΔF, %) of -32.275 ± 5.273 was observed in day 12 samples whereas highest mineral loss (ΔZ, %Vol.µm) was in day 4 samples exposed to the fluoride slurry for 1 hour (494.38 ± 191.71). Differences were statistically significant between day 4 and day 10, 12 and 14 samples as quantified by QLF (p = 0.037, p =

Figure 5.15. Typical QLF-D images of bovine enamel sampled longitudinally within a biological caries model subjected to 100 mM sucrose 8 times daily and 1,450 ppm fluoride continuously. Right side of enamel sample was exposed to experimental conditions; left was protected as an internal control.



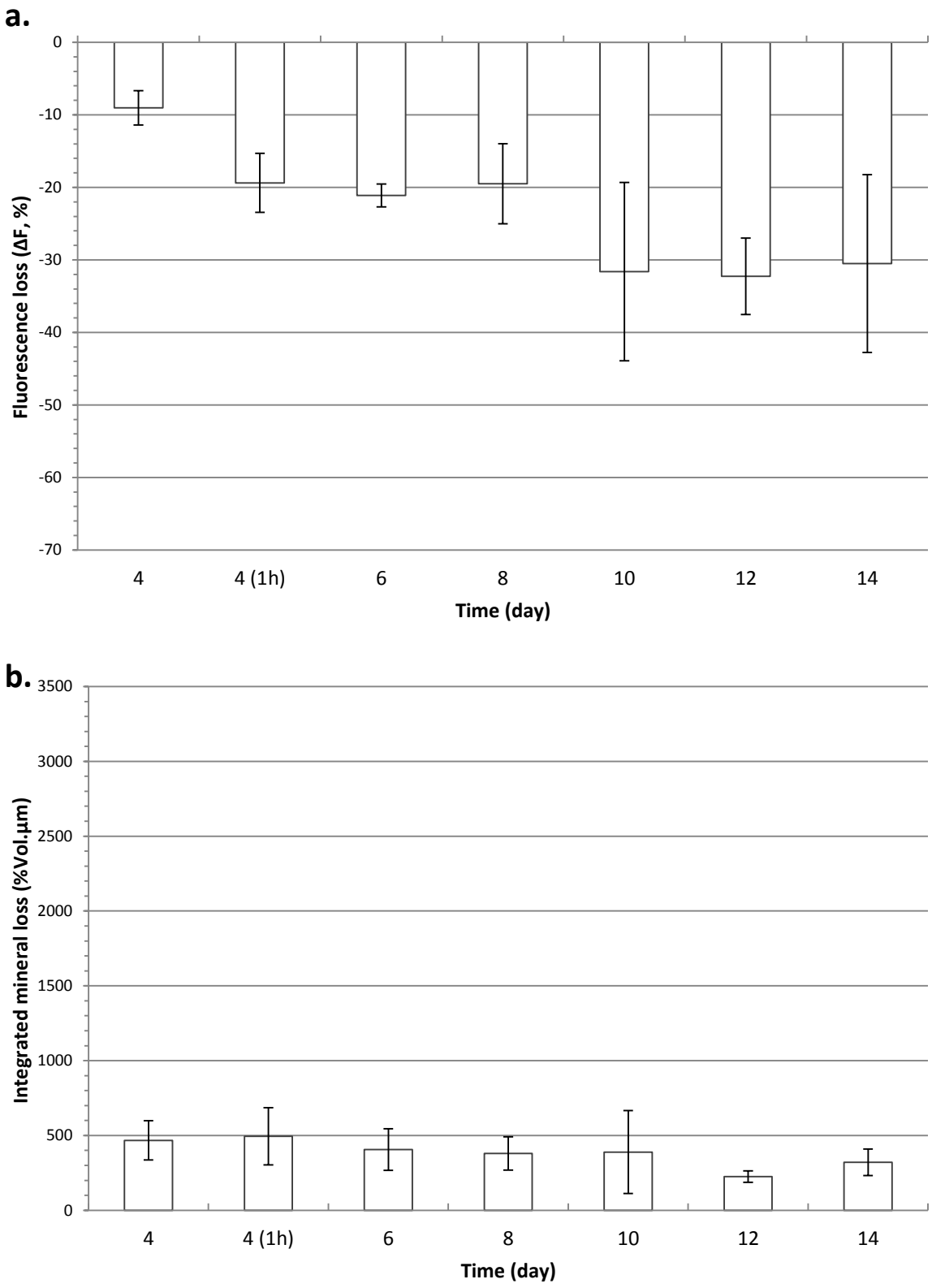
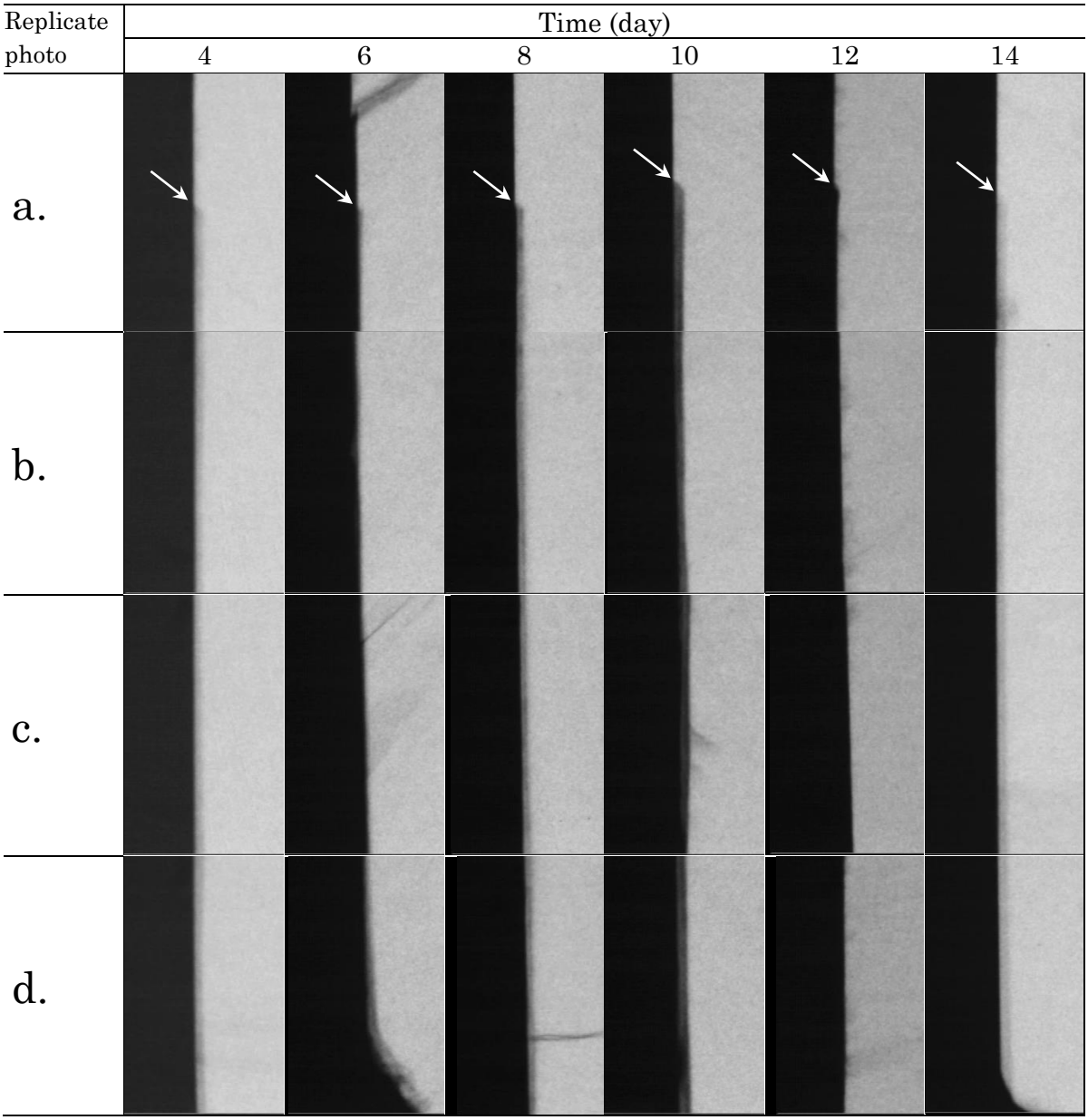


Figure 5.16. Longitudinal changes in (a) enamel fluorescence (average fluorescence loss, ΔF, %) as detected by QLF *in vitro* and (b) mineral loss (average ΔZ, %Vol.µm) determined by TMR in bovine enamel samples exposed to multispecies oral biofilm pulsed with 100 mM sucrose 8 times daily and 1,450 ppm sodium fluoride continuously at a low flow rate. Error bars represent standard deviation, $n = 3$ for ΔF and $n = 2$ for ΔZ.

0.031 and $p = 0.050$ respectively, $n = 3$ in all comparisons). All differences in mineral loss over time were not statistically significant. NCSP detected surface loss throughout the experiment (Figure 5.18) with maximum surface loss in day 14 samples (0.072 ± 0.035 μm, $n = 3$) but all differences over time were not statistically significant. When comparing 1,450 ppm fluoride exposed samples with those exposed to the caries challenge alone (see Chapter 3), ANCOVA adjusting for time provided evidence that differences in ΔF were statistically significant for time but not according

Figure 5.17. Example TMR images of sections of enamel exposed to cariogenic biofilm pulsed with 100 mM sucrose 8 times daily and 1,450 ppm fluoride for up to 14 days. Images were taken equidistant in the direction away (a – d) from the interface between the biofilm exposed and nail-varnish protected areas of the enamel disc (indicated by arrow).



to the presence or absence of fluoride (p = 0.001, F (1, 32) = 62.935 and p = 0.819, F (1, 32) = 0.054 respectively, R^2 = 0.698, n = 18). In terms of ΔZ differences according to fluoride exposure were statistically significant (p = 0.010, F (1, 20) = 8.060) as well as over time and the interaction between the two factors (p = 0.001, F (1, 20) = 29.953 and p = 0.001, F (1, 20) = 44.298 respectively, R^2 = 0.876, n = 12).

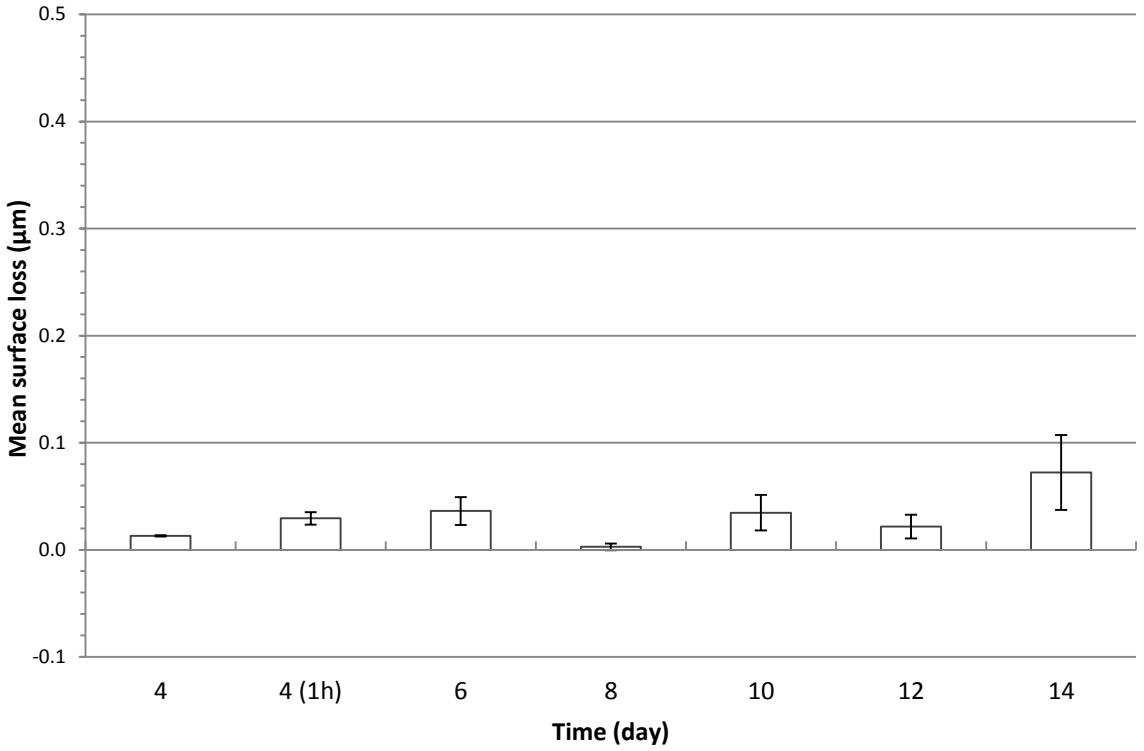


Figure 5.18. Mean surface loss (µm) of bovine enamel samples exposed to 20 mM, 100 mM or no sucrose 8 times daily as detected by NCSP measurements of height differences between negative control and treatment zones within samples. Error bars represent standard deviation, n = 3.

5.3.7 Biofilm formation and growth in the presence of 1,450 ppm fluoride delivered continuously

The microbial composition of multispecies oral biofilm when exposed continuously to 1,450 ppm fluoride along with 8 times daily pulses of 100 mM sucrose is shown in Figure 5.19. Viable counts of total bacteria showed a pseudo-steady state was achieved in all biofilm by day 4. Results indicate that the growth of mutans streptococci was restricted throughout the experiment, reaching pseudo-steady state at 7.44 ± 2.35 SD cfu mm^{-2} (average of day 8 – 14 counts, $n = 4$). Viable counts of *Lactobacillus* spp. at pseudo-steady state were much higher; achieving 4.78 x 10^5 ± 4.52 x 10^5 SD cfu mm^{-2} (average of day 8 – 14 counts, $n = 4$) by day 8. The viable counts of the bacterial constituents of the biofilm before and after 1 hour of exposure to 1,450 ppm fluoride are presented in Table 5.9. The largest percentage change was observed in total *Lactobacillus* spp. Changes were statistically significant ($p < 0.05$) in total bacteria, total streptococci and *Lactobacillus* spp. yet the percentage decrease observed in mutans streptococci was not statistically significant. When comparing microbial members of biofilm exposed to 1,450 ppm fluoride with those in biofilm exposed to the caries challenge alone (see Chapter 3), ANCOVA adjusting for time indicated that differences were statistically significant for total bacteria, total *Streptococcus* spp., mutans streptococci and *Lactobacillus* spp. (Tables 5.10 – 5.13). Time as a factor was only statistically significant in mutans streptococci and *Lactobacillus* spp. populations, suggesting dynamic growth was occurring during the experimental period (Table 5.12 and 5.13). The interaction between time and fluoride addition was statistically significant only in *Lactobacillus* spp. inferring that in this bacterial group, viable counts fluctuated differently over time according to whether fluoride was present or absent.

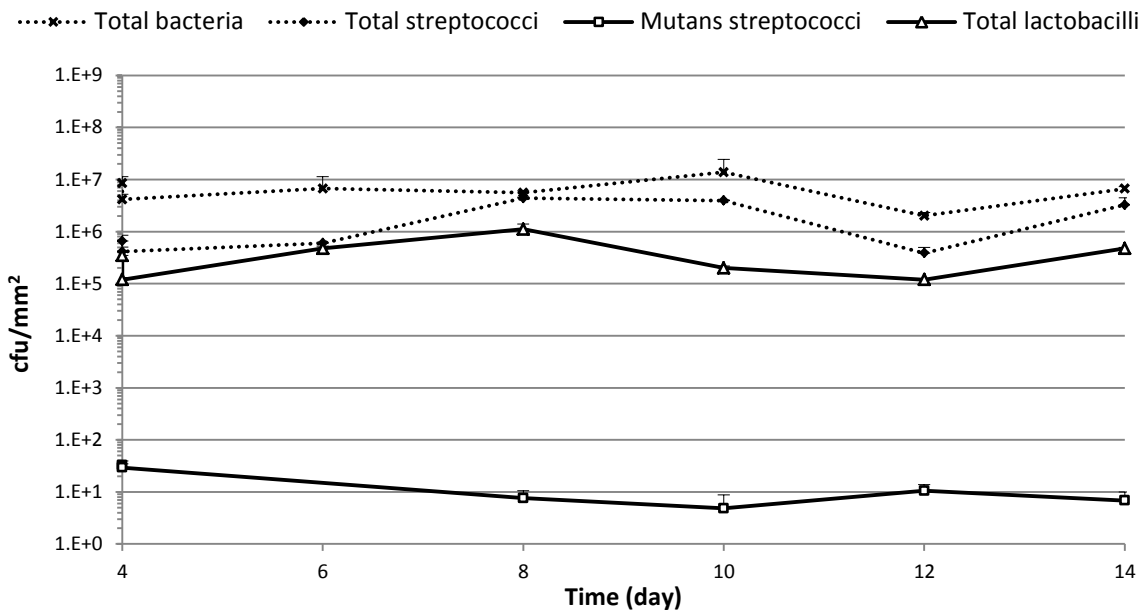


Figure 5.19. Microbial composition of multispecies oral biofilm grown on hydroxyapatite exposed to 1,450 ppm fluoride continuously and pulsed with 100 mM sucrose eight times daily. Error bars represent standard deviation, n = 4.

Table 5.9. Microbial composition of multispecies oral biofilm prior to 1,450 ppm fluoride exposure and one hour after. Change tested for statistical significance using unpaired t-test.

Organism	0 hr (average cfu mm^{-2} ± SD, n = 4)	1 hr (average cfu mm^{-2} ± SD, n = 4)	% change *	t-test *p*-val.
Total bacteria	$8.50 \times 10^6 \pm 2.86 \times 10^6$	$4.14 \times 10^6 \pm 1.11 \times 10^6$	-4.50	**0.013**
Streptococcus spp.	$6.56 \times 10^5 \pm 1.91 \times 10^5$	$4.12 \times 10^5 \pm 1.98 \times 10^4$	-3.47	**0.012**
Mutans streptococci	$3.25 \times 10^1 \pm 2.39$	$2.94 \times 10^1 \pm 7.17$	-2.89	0.198
Lactobacillus spp.	$3.49 \times 10^5 \pm 1.54 \times 10^5$	$1.19 \times 10^5 \pm 1.90 \times 10^4$	-8.42	**0.005**

* of $\log_{10}$ transformed bacterial counts

bold font denotes difference is statistically significant.

Table 5.10. Results of ANCOVA testing statistical significance of time and fluoride addition in **total viable bacteria*** exposed to 100 mM sucrose and either **no or 1,450 ppm fluoride continuously**.

Source of Variation	SSIII	*df*	MS	F	*p*
Fluoride	303.046	2	151.523	658.787	**< 0.001**
Time	0.000	1	0.000	0.001	0.975
Fluoride x time	0.298	1	0.298	1.295	0.261

R Squared = 0.996 (Adjusted R Squared = 0.995)

Table 5.11. Results of ANCOVA testing statistical significance of time and fluoride addition in **total *Streptococcus* spp.*** exposed to 100 mM sucrose and either **no or 1,450 ppm fluoride continuously**.

Source of Variation	SSIII	*df*	MS	F	*p*
Fluoride	264.090	2	132.045	399.093	**< 0.001**
Time	0.055	1	0.055	0.166	0.686
Fluoride x time	0.484	1	0.484	1.464	0.233

R Squared = 0.993 (Adjusted R Squared = 0.993)

Table 5.12. Results of ANCOVA testing statistical significance of time and fluoride addition in **mutans streptococci*** exposed to 100 mM sucrose and either **no or 1,450 ppm fluoride continuously**.

Source of Variation	SSIII	*df*	MS	F	*p*
Fluoride	77.563	2	38.782	94.071	**< 0.001**
Time	2.201	1	2.201	5.339	**0.027**
Fluoride x time	0.033	1	0.033	0.079	0.780

R Squared = 0.968 (Adjusted R Squared = 0.965)

Table 5.13. Results of ANCOVA testing statistical significance of time and fluoride addition in **total *Lactobacillus* spp.*** exposed to 100 mM sucrose and either **no or 1,450 ppm fluoride continuously**.

Source of Variation	SSIII	*df*	MS	F	*p*
Fluoride	140.385	2	70.192	485.874	**< 0.001**
Time	7.531	1	7.531	52.128	**< 0.001**
Fluoride x time	10.743	1	10.743	74.366	**< 0.001**

R Squared = 0.996 (Adjusted R Squared = 0.996)

Type III sum of squares, SSIII; degrees of freedom, *df*; mean square, MS; F = (MS between groups)/(MS within groups). *$\log_{10}$ transformed prior to analysis. **bold** font denotes difference is statistically significant.

5.3.8 Surface characterisation of biofilm demineralised enamel and fluoride deposition on samples exposed to 1,450 ppm fluoride

SEM revealed the smooth surface of sound bovine enamel with slight indications of characteristic interprismatic morphology (Figure 5.20a). EDX analysis of enamel exposed to the multispecies biofilm for 4 days elucidated the loss of phosphorus (13.77 vs 16.20 wt. %) and a slight increase in calcium (30.37 vs 30.53 wt. %) compared to control regions of enamel, protected from the effects of the experimental conditions (Figure 5.22 and Table 5.14). After 1 hour of exposure to the fluoride solution an increase in phosphorus (14.24 vs 11.64 wt. %) and decrease in calcium (28.72 vs 30.14 wt. %) were observed (Figure 5.22 and Table 5.14) whilst the presence of fluoride on the surface of these samples was below the detection limits of the technique. The surface of bovine enamel after exposure to cariogenic multispecies biofilm pulsed with 100 mM sucrose 8 times daily for 14 days and 1,450 ppm fluoride continuously from day 4 onwards, was substantially more textured than sound enamel (Figure 5.20b). Calcium fluoride-like deposits of varying sizes were observed. The majority of deposits appear to be small (5.6 μm to 11.1 μm diameter) and medium (16.7 μm) in size whilst a small number of large globules were also present (50.0 μm). Structures smaller than this are most likely the ends of rounded crystals within the enamel; whilst no distinct intercrystalline spaces, rod and interrod dissolution or emergence of perikymata were elucidated, supporting the finding that surface loss was minimal. At lower magnifications, larger areas of joined mass were apparent with texture suggesting they may be areas where globules merged, enamel remineralised or areas of less surface demineralisation (Figure 5.21). EDX revealed that by day 14 (10 days exposure to fluoride), the loss of phosphate in enamel was substantial (4.86 vs 13.59 wt. %) whilst ample fluoride was detected on enamel surfaces compared to the area of enamel protected from the conditions of the experiment. Furthermore, differences in calcium between exposed and unexposed sides within each enamel sample were minute at day 14 (35.88 vs 34.63 wt. %).

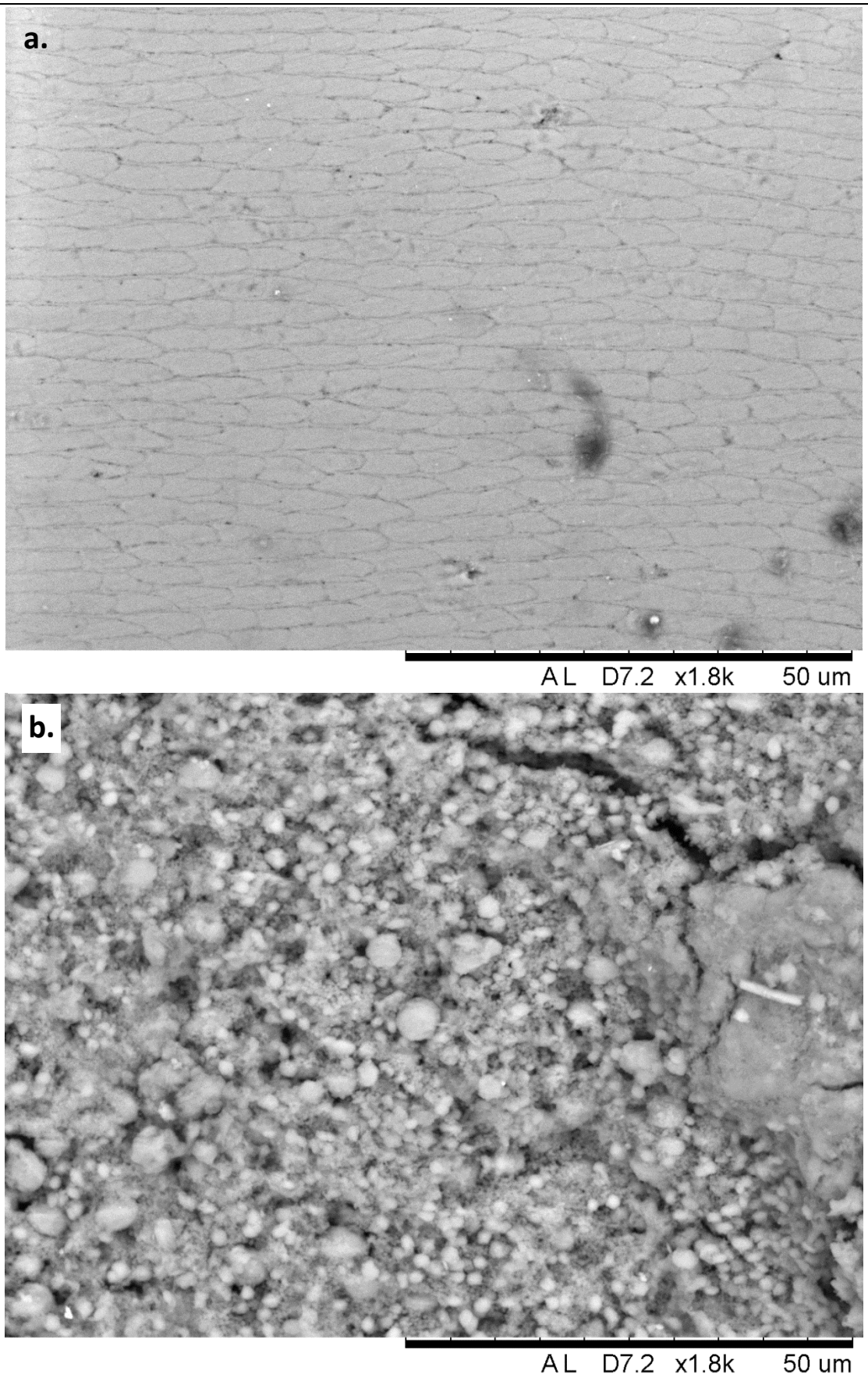


Figure 5.20. Scanning electron microscopy images of the surface of (a) sound bovine enamel and (b) enamel exposed to cariogenic biofilm pulsed with 100 mM sucrose 8 times daily for 14 days and exogenous 1,450 ppm fluoride continuously from day 4 onwards.

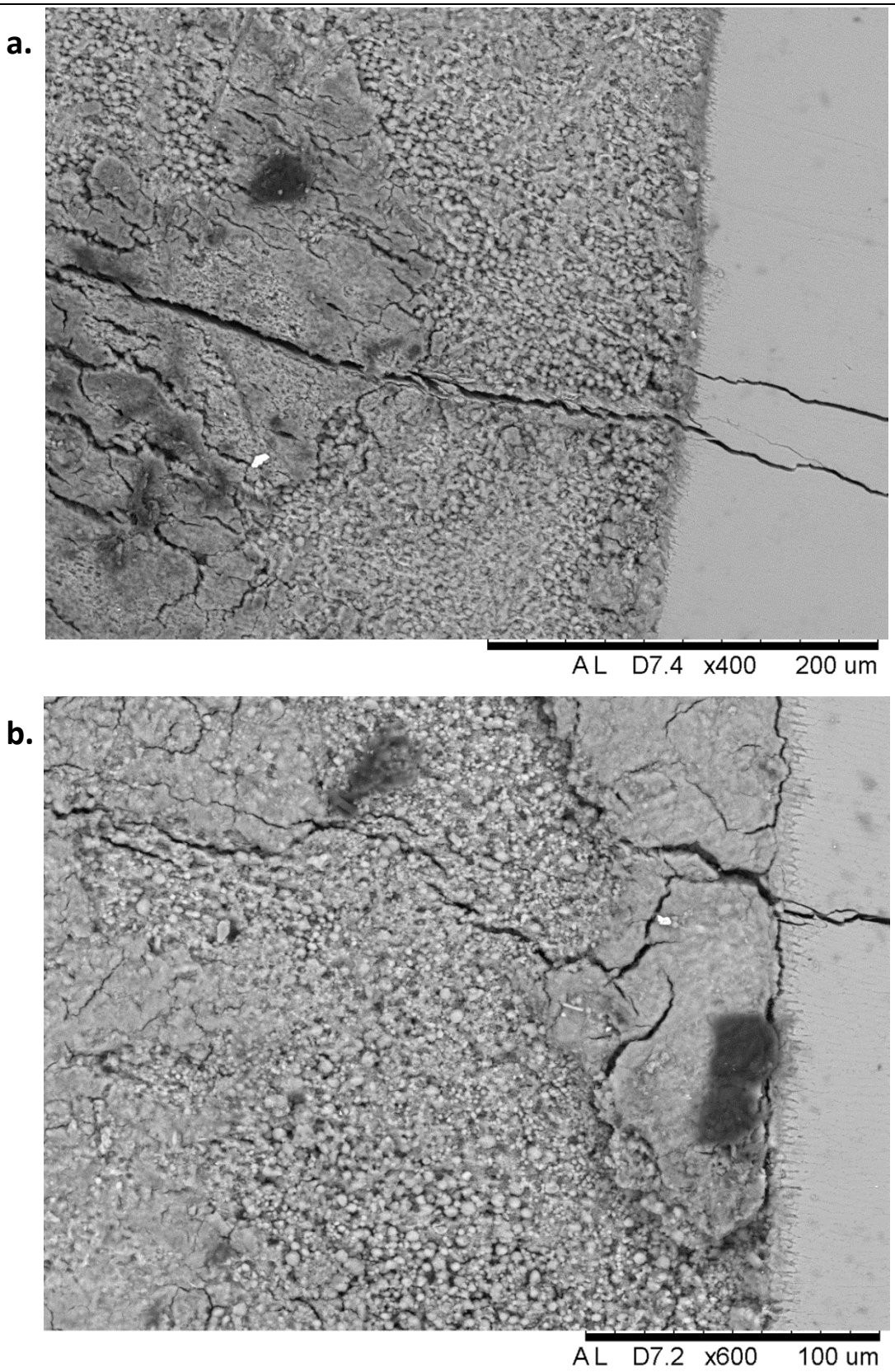


Figure 5.21. Scanning electron microscopy images of the surface of bovine enamel exposed to cariogenic biofilm pulsed with 100 mM sucrose 8 times daily for 14 days and exogenous 1,450 ppm fluoride continuously from day 4 onwards. (a) x400 and (b) x600 magnification.

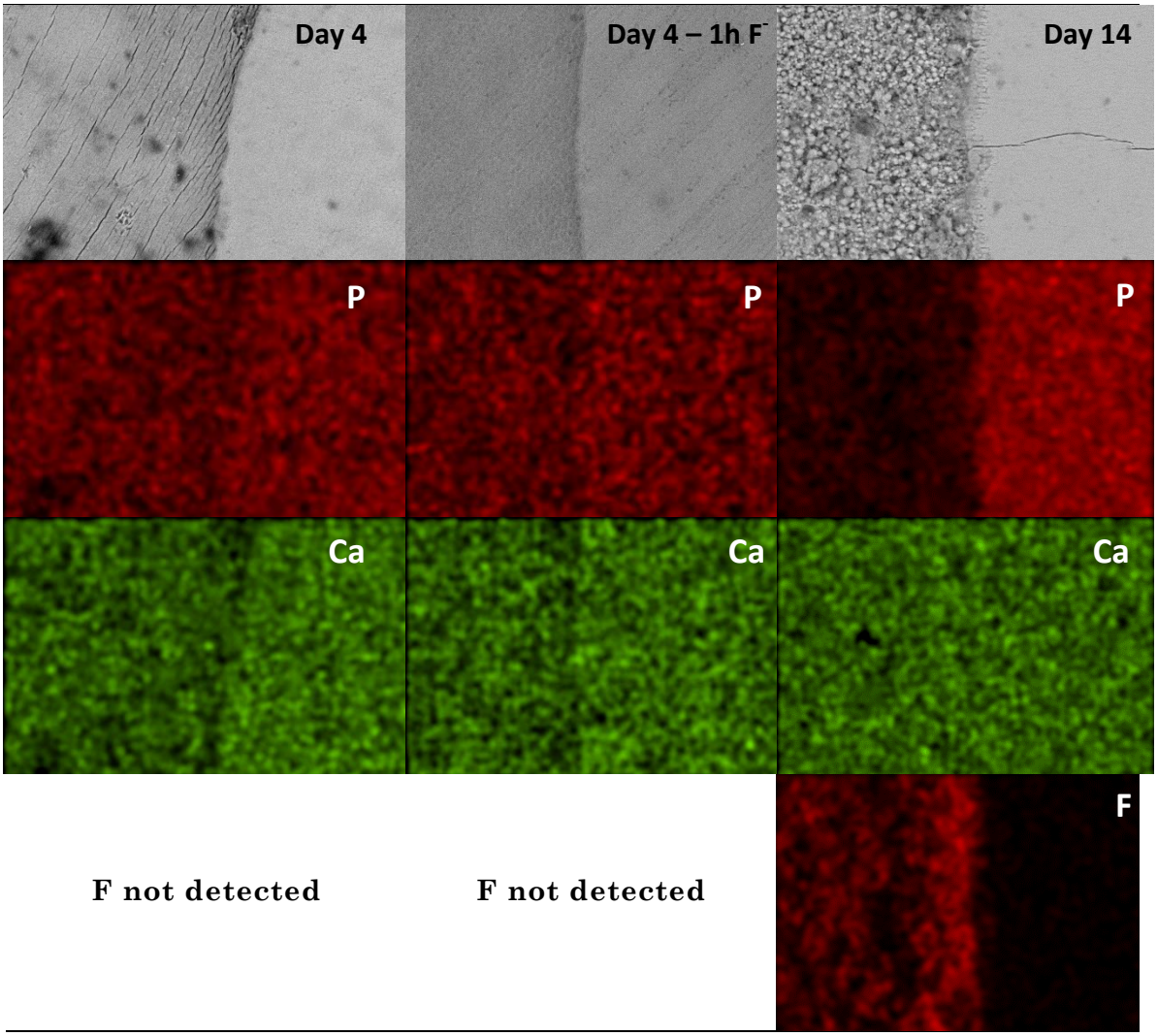


Figure 5.22. Scanning electron microscopy images of the surfaces of bovine enamel exposed to cariogenic biofilm pulsed with 100 mM sucrose 8 times daily for 14 days and 1,450 ppm fluoride from day 4 onwards. EDX images reveal the elemental composition and specific location across each surface. Here profiles for phosphorus (P), calcium (Ca) and fluoride (F) are included.

Table 5.14. Elemental composition (wt. %) of bovine enamel exposed to multispecies cariogenic biofilm for 4 days and no fluoride, for 4 days and 1 hour of 1,450 ppm fluoride and for 14 days and 1,450 ppm fluoride from day 4 onwards; compared within sample non-exposed enamel as control.

	Day 4 0 h fluoride		Day 4 1 h fluoride		Day 14 10 days fluoride	
Element	control	exposed	control	exposed	control	exposed
Phosphorus	16.20	13.77	11.64	14.24	13.59	4.86
Calcium	30.37	30.53	30.14	28.72	35.88	34.63
Fluoride	0.00	0.00	0.00	0.00	2.80	22.54

5.4. DISCUSSION

The previously developed dual CDFF model (see Chapter 4) , enabled the direct comparison between biofilm begat from the same microbial inoculation process and exposed to the same cariogenic challenge. In order to effectively investigate the influence of continuous exposure to fluoride at low concentration compared to non-exposed 'controls'. Whilst the singular CDFF approach (Chapter 3) elucidated the effects of continuous exposure to high concentration fluoride on caries progression. The experiments of this study indicated that continuous exposure to 0.05 ppm fluoride did not diminish enamel demineralisation but lead to the formation of laminated zones within the resultant caries lesions by the tenth day. This study also revealed that continuous exposure to 1,450 ppm sodium fluoride slurry exerted an anti-caries effect on enamel beneath multispecies biofilm. A small degree of surface erosion was observed but this was arrested over time. Continuous exposure to fluoride differentially influenced the microbial dynamic of mutans streptococci and *Lactobacillus* spp. within multispecies biofilm compared to non-fluoride exposed biofilm depending on its concentration.

5.4.1 Growth of bacterial members of multispecies biofilm under caries challenge in the presence of continuous fluoride

Continuous exposure to fluoride; be it low (0.05 ppm) or high (1,450 ppm); significantly affected the growth of total bacteria, *Streptococcus* spp., mutans streptococci and *Lactobacillus* spp. within multispecies biofilm compared to non-fluoride exposed biofilm. On a whole it would appear that in the present study fluoride differentially suppressed the growth of bacterial groups commonly associated with caries; mutans streptococci and *Lactobacillus* spp. Estimated marginal means analysis, discounting time from the statistical model, elucidated that differences in *Lactobacillus* spp. and mutans streptococci were statistically significant when fluoride was added. Early work showed that 100 ppm (5.26 mM) fluoride was required to

reduce growth in pure cultures of various oral acidogenic bacteria, (Bibby and van Kesteren, 1940) and in mixed cultures of salivary organisms (Wright and Jenkins, 1954). Though cellular membranes (Yotis *et al.*, 1983) and cytoplasmic proteins (Kashket and Bunick, 1978; Katayama *et al.*, 1981) can bind fluoride, significant differences observed in the binding of ^{18}F among a number of oral bacteria indicates clear differences in either the number of F-binding sites or the affinity of the existing sites for fluoride (Yotis and Brennan, 1983). This suggests that fluoride should exert a differential effect on the composition of dental plaque but in reality, while high concentrations of fluoride administered topically may bear selective properties on the microflora of dental plaque (Loesche *et al.*, 1973; Loesche *et al.*, 1975; Brown *et al.*, 1983), naturally occurring fluoride has not been shown to effect dental plaque composition in this way, even at concentrations as high as 1 mM (19 ppm) F^- (Kilian *et al.*, 1979; Bowden *et al.*, 1982). Despite the early *in vitro* evidence that fluoride at extremely low concentrations can inhibit bacterial growth (Maltz and Emilson, 1982; Milnes *et al.*, 1985), the levels of fluoride achieved *in vivo* are insufficient to inhibit growth, except at the high levels associated with typical applications of topical fluoride. Evidence indicates that the fluoride sensitivity of various genera and species is markedly varied and influenced by the acidic conditions of the plaque environment. *S. mutans*, for example, was found to be 20- to 40- times more sensitive than *Lactobacillus casei* and 7-times more sensitive than some species of *Actinomyces* (Hamilton and Bowden, 1988). Fluoride also inhibited the growth of bacterial suspensions of *A. naeslundii*, *A. oris*, *S. mutans* and *S. sanguinis* in a dose dependent manner though the growth of *Actinomyces* was shown to be more tolerant to the effects of fluoride (Kawashima *et al.*, 2013). The sensitivity of bacteria to fluoride greatly increased as pH fell (Shiota, 1956; Jenkins, 1959).

The proportion of mutans streptococci was most supressed throughout exposure to the sucrose challenge and 1,450 ppm fluoride, save for the first hour. It was shown previously (see Chapter 3; Section 3.3.4) that 100 mM sucrose pulsed onto multispecies biofilm 8 times daily supressed the growth of mutans streptococci. Therefore, the fluoride exerted major additive effects on the cariogenicity of this bacterial group since, in its presence, viable counts of mutans streptococci were lowest. After 1 hour exposure to the sodium fluoride slurry the lowest percentage change was observed in mutans streptococci and the decrease was found not to be statistically significant. This may indicate that of the biofilm constituents quantified, this bacterial group may possess mechanisms to resist the antibacterial effects of fluoride but, that these mechanisms eventually failed in the prolonged presence of such a high concentration of fluoride. However, in biofilm exposed to 0.05 ppm fluoride continuously, mutans streptococci were markedly lower than in the presence of no fluoride. By day 8 it appears pseudo-steady states were achieved under both conditions but by day 14 it appears the proportions of mutans streptococci were higher in the presence of 0.05 ppm fluoride than in its absence. Fluoride-resistant bacteria have been reported previously *in vitro* in oral streptococci (Williams, 1964) as well as in lactobacilli (Green and Dodd, 1957) and using animal models (van der Hoeven and Franken, 1984). Brown observed that *S. mutans* and *Lactobacillus* species were not all eliminated in the plaque of xerostomic patients over a 5 year period despite the slow clearance by saliva of, and daily application of, 1% sodium fluoride gels, though it is presumed that enough bacterial cells remained susceptible since acid production continued to reduce regardless (Brown *et al.*, 1981).

Conversely, in 1,450 ppm fluoride exposed biofilm counts of *Lactobacillus* spp. were only slightly lower at steady state in fluoride exposed biofilm ($4.78 \times 10^5 \pm 4.52 \times 10^5$ SD cfu mm^{-2}) than in non-fluoride sucrose only exposed biofilm ($1.46 \times 10^6 \pm 2.59 \times 10^5$

SD cfu mm^{-2}). However, since this an assessment between sequential CDFF experiments caution in the interpretation of this comparison must be taken. Nonetheless, this could be an indicator of the additive inhibitory effect of fluoride on *Lactobacillus* spp. and lower counts of this bacterial group could be an indicator of the lowered acidity of the biofilm in the presence of fluoride. On the other hand, their persistence in comparison to counts obtained under continuous exposure to 0.05 ppm fluoride could also serve as an indicator of the acidity of the environment as a consequence of the marginally acidic 1,450 ppm sodium fluoride slurry. Statistically significant differences according to time when comparing numbers of *Lactobacillus* spp. and mutans streptococci in the presence or absence of 1,450 ppm fluoride reveal that the growth of these bacterial groups are highly dynamic in response to sucrose and high concentration fluoride. Furthermore, statistical significance in the interaction of exposure to 1,450 ppm fluoride and time suggest the growth of *Lactobacillus* spp. differed in markedly different manors at each time depending on the presence or absence of high concentration fluoride. Conversely, the significant interaction between fluoride and time for mutans streptococci and for *Lactobacillus* spp. exposed to 0.05 ppm fluoride suggests that the growth trend of both bacterial groups over time differed markedly dependent on this low concentration of fluoride.

The growth of *Lactobacillus* spp. was most markedly suppressed under conditions of high frequency sucrose pulsing along with the continuous presence of 0.05 ppm fluoride (detectable only at day 10; 2.76 ± 0.98 SD cfu mm^{-2}). Viable counts of this bacterial group were over a million-fold higher in the absence of 0.05 ppm fluoride ($9.08 \times 10^6 \pm 9.77 \times 10^4$ SD cfu mm^{-2} at day 10). As discussed, members of *Lactobacillus* spp. are highly acid tolerant and have demonstrated better resistance to fluoride in acidic environments than other bacteria such as *S. mutans* (Hamilton and Bowden, 1988). Therefore it is unlikely that the growth of *Lactobacillus* spp. in this

study was modulated by the effect of low level fluoride in combination with the sucrose challenge alone. In ideal circumstances replication of this experiment would fully corroborate the repeatability of this result. It is possible that factors inherent in traditional microbiological techniques such as the formulation of the batch of culture plates, co-aggregation of bacterial cells, and conditions of incubation were not met. Nonetheless, the lower bacterial counts could be attributed to the increased presence and combination of ions released into the oral environment as enamel demineralised, such as calcium, hydroxyl and phosphorus ions. The presence of lamination zones in the underlying enamel of biofilm exposed to 0.05 ppm fluoride indicates that available fluoride was incorporated into various depths of the lesion body, discussed thoroughly later. It has been emphasised that the degree of saturation of specific ions in the plaque fluid of oral biofilm is an important factor to consider in assessing its cariogenicity and that this medium reflects the chemical changes induced by microbial activity whilst existing in close contact with the enamel surface (Margolis, 1990). Little has been previously described on the influence of ions released from enamel during the caries process on the viability of the microorganisms of dental plaque, not to mention their effects on *Lactobacillus* spp. in particular. However, it has been noted that calcium ions could promote bacterial attachment and aggregation to teeth by binding to acidic groups in plaque (Rose *et al.*, 1993). The adherence of the periodontopathic bacterium *Porphyromonas gingivalis* was influenced in particular by divalent calcium bridges than other oral bacteria including gram positive cariogenic bacteria (Yamashita *et al.*, 1991). It is thought that calcium bound to bacterial cell walls may be released into the plaque fluid during a cariogenic challenge and may reduce the driving force for mineral dissolution triggered by a drop in pH (Leitao *et al.*, 2012). Furthermore, calcium hydroxide, through its chemical dissociation into constituent calcium ions and hydroxyl groups, possesses antibacterial effects in studies of endodontic dressing (Estrela *et al.*, 1995) and of its effects on *Candida albicans* (Waltimo *et al.*, 1999). Its comparative activity as an antibacterial has been

demonstrated (Bystrom *et al.*, 1985). The principal antibacterial action appears to be due to the effect of the hydroxyl ions on the permeability and transfer of substances through the bacterial cytoplasmic membrane and inhibiting bacterial enzymes (Estrela *et al.*, 1994). For bacteria enzymatic systems inhabit elemental roles in metabolism, cellular growth and division and each enzyme possesses an optimum pH wherein the velocity of its reactions are maximised (Lehninger *et al.*, 2013). At severely non-optimal pH enzymes are inactivated (Lehninger *et al.*, 2013) but it has been shown that this process can be reversible upon a return to favourable pH (Kodukula *et al.*, 1988). Calcium hydroxide also possesses the ability to influence the mineralisation of dental tissue by activating the tissue enzyme alkaline phosphatase and leading to mineralisation (Binnie and Mitchell, 1973; Tronstad *et al.*, 1981). It appears this mineralisation activity was not effective in the context of the current study and may be due to the low baseline pH of the biofilm exposed to 0.05 ppm and the lack of sufficient enzyme source *in vitro*. The optimal pH range for the activation of alkaline phosphatase is from 8.6 to 10.3 and varies with temperature, the type and concentration of substratum and with the source of enzymes (Tronstad *et al.*, 1981). The baseline biofilm pH within the present study was no higher than 5.66. Therefore, any calcium hydroxide present could not overcome the continual production of acids by the microbial members of the oral biofilm. It is also possible that complex microbial interactions in response to low level fluoride within the multispecies biofilm developed in the current study were antagonistic or non-conducive for the growth of *Lactobacillus* spp. For example, the recovery and dynamic growth over time of mutans streptococci, evidently more suited to the conditions of the experiment, may have influenced the availability of resources for other members of the biofilm community.

5.4.2 Effects on enamel demineralisation beneath multispecies biofilm under caries challenge in the presence of continuous fluoride

Regardless of conflicting views regarding the significance of the bactericidal role of fluoride in comparison to its role on protecting against enamel demineralisation and in favouring remineralisation (see Section 1.5.3), its measurable inhibition of acid production is nonetheless important in the larger context of its anti-caries abilities. Findings of the current study agree with previously reported observations of the impact of low and high levels of fluoride on acid production and enamel exposed to caries simulating conditions. Though the current study cannot directly discriminate between these effects it is nonetheless highly likely that both its enamel- and acid production- related effects contributed. Elsewhere, support for the antibacterial effect of topical fluoride was evident when a differential degree of demineralisation was observed in enamel by *S. mutans* biofilm exposed to either fluoride, nigericine or neither(van Loveren *et al.*, 1987). Like fluoride, nigericine reduces the difference between the intracellular and extracellular pH of bacterial cells yet without any effect on the solubility of enamel. In this *in vitro* study, microradiographic profiles were captured and the level of calcium dissolving from the enamel was measured. Of the three test scenarios, a markedly higher level of protection was observed in samples exposed to fluoride. The amounts of enamel dissolved were inhibited by 89% and 68% in the fluoride and nigericine groups respectively, suggesting that inhibition of acid production may be an underestimated aspect of fluoride's activity. Early work showed that 1 – 2 ppm (52.6 – 105.2 μM) fluoride had a detectable effect on acid production but 10 ppm (526 μM) were needed for decisive inhibition in pure cultures of various oral acidogenic bacteria, (Bibby and van Kesteren, 1940; Murray *et al.*, 1991) and in mixed cultures of salivary organisms (Wright and Jenkins, 1954). An *in vivo* study revealed that when microcosm plaque bacteria were exposed for 10 minutes to 225 and 900 (47.4 mM) ppm F^-, lactate production from 10% (555 mM) glucose was

inhibited by 34% and 46%, respectively (Takahashi and Washio, 2011). Later it was shown in an *in vitro* study that systems involved in acid production and acid tolerance in biofilms of *S. mutans* appear to be more susceptible to fluoride than systems of EPS synthesis (Pandit *et al.*, 2013). Fluoride concentration was found to be closely related to its activity in affecting acid production in *S. mutans* biofilms *in vitro* and that the relationship followed a sigmoidal dose-response pattern (Pandit *et al.*, 2013). Here a reduction in the initial proton production levels was observed when biofilm was exposed to concentrations from 10 ppm (0.526 mM) F^- with maximum efficacy at concentrations including and over 30 ppm (15.8 mM) F^-. Acid production was completely inhibited when saliva adjusted to pH 5.0 was incubated with sugar and fluoride solutions of concentrations of 6 ppm (316 μM) and over. In fact pH rose due to the continued unopposed bacterial production of bases (Jenkins, 1959). The daily use of a fluoride rinse lead to reduced plaque acidogenicity in the presence of sucrose and increased plaque fluoride concentration but this anti-caries effect was lost once use was ceased (Geddes and McNee, 1982) and acid production fell in xerostomic patients using fluoride containing gel over a 5 year period (Brown *et al.*, 1981). Increased proton permeability was elucidated from 10 ppm F^- with maximum efficacy at concentrations including and above 300 ppm (15.8 mM) F^-. The activity of a key determinant of acid tolerance; F-ATPase; (Lemos and Burne, 2008); was also affected when concentrations of fluoride were high; at 100 and 300 ppm F^- (Pandit *et al.*, 2013). Finally, reductions in acidogenicity could be indicative of cell protection against fluoride since the fluoride sensitivity of cells is potentiated by increased H^+ in the environment (Kashket *et al.*, 1977).

Since the low level of fluoride tested in the present study (0.05 ppm) was significantly below the concentrations determined as effective in reducing acid production it is understandable that the present findings distinguished no statistical difference

between lesions developed in its presence or its absence. Multiple measurements of pH across a number of biofilm also demonstrate the variability and highly distinct micro-environments as a result of differential metabolism by members of the microcosm dental plaque. On the other hand, no significant caries lesions were observed in enamel beneath biofilm exposed to the sucrose challenge and continuous 1,450 ppm fluoride. This could partially be attributed to the reduced numbers and acidogenicity of mutans streptococci and *Lactobacillus* spp. observed as well as, and more likely due to, the effects of fluoride on reducing enamel solubility and encouraging remineralisation as discussed in Section 1.5. However, classic arrested surface erosion craters extending no more than 10 μm were observed at later days, whilst some surface softening of no more than 20 μm deep was visible at earlier days, in the experimental run. Subsequent statistical testing revealed that there was no significant increase or decrease in erosion over time since all differences in mineral loss over time were not statistically significant. NCSP detected surface loss throughout the experiment with maximum surface loss in day 14 samples but all differences over time were also not statistically significant. The effect of fluoride was not statistically significant according to ΔF but was significant in terms of TMR ΔZ and lesion depth. The statistically significant interaction between the factor of fluoride presence/absence and time indicates that the mineral loss and lesion depths differ in different ways in the presence of fluoride compared to sucrose alone. In conclusion it appears QLF quantified demineralisation in a distinctly different way compared to TMR. Largest fluorescence change, as an indication of mineral loss, was in day 12 whereas for TMR, mineral loss was greatest at day 4; 1 hour after fluoride flow was initiated. This disparity between the techniques could be attributed to the wall effect of erosive lesion types on QLF measurements.

A number of theories exist surrounding the mechanisms of enamel autofluorescence and its decrease in demineralised enamel (Angmar-Månsson and ten Bosch, 2001). It has been theorised that a greater degree of light-scattering occurs within demineralised enamel than in sound enamel meaning that the light path within the lesion is shortened and absorption of light is lower resulting in lower levels of fluorescence (ten Bosch, 1996). The light scattering within demineralised enamel could also prevent the penetration of light to and from the underlying enamel-dentine junction (EDJ). Elsewhere, it was proposed that the demineralisation process could cause a change in the molecular environment of the chromophore leading to a decrease in fluorescence (Spitzer and Bosch, 1976) or it could simply be that during demineralisation chromophores are lost. Conflicting theories have mainly surrounded the role of the underlying dentine and in particular the enamel-dentine junction (EDJ); most investigators appear to believe that the enamel fluorescence is due to the EDJ (Amaechi and Higham, 2002). However, Rousseau *et al.* (2002) reported that the EDJ is not required for the accurate assessment of demineralisation by QLF in bovine enamel. Nonethless, using Monte Carlo simulations, it has been shown that the phenomenon of light scattering most suitably explains the mechanism of QLF (van der Veen *et al.*, 2002). It has been theorised that changes in fluorescence quantified by QLF can be attributed to the light scattering effect of carious enamel which prevents penetration of the excitation energy to the underlying dentine (Ko *et al.*, 2000; van der Veen *et al.*, 2002) and that enamel thickness also plays a part (Ando *et al.*, 2003). It was subsequently derived that a combination of lesion softening and frank erosive mineral loss lead to walls which could prevent excitation energy from reaching the enamel surface and that the walls hinder the release of fluorescing light due to enumerable scattering effects (Pretty *et al.*, 2004). This theory plausibly explains the higher decreases of fluorescence loss observed compared to TMR mineral loss observed in the current study. Additionally it explains why no significant differences were found when comparing ΔF in samples with or without fluoride

exposure. The significant differences in ΔZ and lesion depth reflect that minimal caries developed in samples exposed to 1,450 ppm fluoride continuously compared to non-fluoride samples exposed to sucrose pulsing alone.

Since craters were most prominent at the junction between nail-varnish protected and unprotected regions of each disc it is likely that this pattern of erosion is due to the accumulation of acidic products at this interface. Ordinarily within the caries process, the surface enamel is more resistant to dissolution. It would appear that fluoride at this concentration was protective of subsurface demineralisation and that it arrested ongoing erosion but the acidity of the sodium fluoride solution itself could have attributed to the erosive effect of the model parameters on a whole. The finding that the highest mineral loss was in day 4 samples exposed to the fluoride slurry for 1 hour and did not significantly develop over time supports these interpretations. Evidence of a classic caries lesion was found in only one enamel disc sampled at day 10, three sections of which contained this lesion and the other did not. This highlights the potential for caries in the model and is evidence of the heterogeneous nature of biological models of caries. It is surprising that only one disc of the three sampled on this day possessed caries lesions and could be due to a number of factors. The prime being that fluoride could not properly exert its anti-caries effects on this particular individual biofilm and enamel within the sample pan. Caries is a multifactorial process and any factor can play its part in predisposing towards enamel demineralisation. The solution may not have accessed this sample or the fluoride may not have penetrated the biofilm as effectively as at other time-points or for other samples. Nonetheless, subsurface demineralisation at this time-point was small and mineral loss was significantly lower than if no fluoride was present. Overall, it would appear that continuous exposure to 1,450 ppm fluoride inhibited subsurface dissolution when enamel was exposed to a highly cariogenic sucrose pulsing

challenge. Prolonged continuous exposure to this slight acidic solution may well have contributed to small degrees of surface softening and frank surface loss but this was minimal and the fluoride arrested the development of this erosive phenomenon over time.

According to both QLF and TMR, demineralisation appeared to be greater in 0.05 ppm fluoride exposed enamel compared to samples exposed to the sucrose pulsing regime alone. The progression of caries over time was confirmed in samples beneath biofilm developed in the presence or absence of 0.05 ppm fluoride and the sucrose challenge when differences in ΔF, ΔZ and lesion depth between samples from day 4 and 14 were statistically significant within each condition. TMR lesion profiles confirmed that there was subsurface demineralisation at day 10 and 14 and none at day 4 under both conditions. Nonetheless, enamel demineralisation over time was more clearly demonstrated according to all three descriptors of caries in 0.05 ppm fluoride exposed samples as supported by statistical significance according to multiple comparisons testing over time. This finding suggests that enamel demineralisation was not only more distinct but speedier in the presence of this level of fluoride. The shape of lesion profiles, confirmed by TMR images, of day 10 and 14 samples, indicated that a degree of surface softening occurred simultaneous to the caries process under both conditions. By day 14, the average subsurface demineralisation zone could be clearly distinguished regardless of surface softening in lesions in enamel exposed to the caries challenge alone, while in those exposed to added fluoride the demineralisation zone was broad with less mineral intact at the enamel surface. However, TMR did not elucidate a response to the addition of 0.05 ppm fluoride since ΔZ and lesion depths were not significantly different compared to non-fluoride exposed enamel at all time-points. Meanwhile, when comparing ΔF in the presence or absence of 0.05 ppm fluoride at each time point, fluorescence loss and thus mineral loss,

differed significantly in day 14 samples. Regardless, the key finding is that 0.05 ppm fluoride did not play a significant anti-caries role when enamel is simultaneously undergoing a significant caries challenge. No significant levels of remineralisation were detected in this group compared to if no fluoride was present. However, statistical significance in ΔF for the interaction of fluoride with time reveals that changes in fluorescence loss differed over time depending if fluoride was present or not, indicating that fluoride at low levels may exert subtle effects on enamel mineralisation.

It has been shown that the use of fluoride products causes an immediate increase in fluoride concentration in saliva before decreasing with time as fluoride is cleared from the mouth however, fluoride can be retained at concentrations in the saliva between 0.03 and 0.1 ppm for 2 – 6 hours depending on the product and the individual (Bruun *et al.*, 1984; Zero *et al.*, 1992). Clinically it has been shown that mean salivary levels in 7 – 12 year old children were approximately 0.05 ppm fluoride regardless of residence in communities with fluoridated or non-fluoridated water (Shields *et al.*, 1995; Shields *et al.*, 1997). The role of low levels of background fluoride in the prevention of caries has previously been noted (Featherstone, 1999). Evidence shows that low but slightly elevated levels of fluoride in saliva and plaque help prevent and reverse caries by inhibiting demineralisation and enhancing remineralisation. To date, there have been no other studies evaluating the caries preventative impact of 0.05 ppm fluoride using CDFF model approaches. Using a pH cycling model, it was shown that remineralisation was enhanced in enamel bathed with artificial saliva with levels of 0.03 ppm fluoride or higher (Featherstone *et al.*, 1986; Featherstone *et al.*, 1990; Featherstone and Zero, 1992). Significant remineralisation was not observed in the current study despite being slightly higher than 0.03 ppm in concentration. This may be attributable to the lack of a biofilm in pH cycling models

and that the demineralisation parameters in these studies were not as conducive of caries as those of the present model. The absence of biofilm means pH cycling models do not fully represent the mixture of acids formed by biofilm bacteria in the natural caries process and inherently underestimate the requirement of fluoride to penetrate in order to exert its beneficial effects in favouring remineralisation and decreasing enamel solubility. Nonetheless, Featherstone *et al* observed a log-linear relationship was revealed between fluoride concentration and degree of protection against caries-like demineralisation and concluded that an optimum of remineralisation was at approximately 0.08 ppm. This has been further demonstrated in clinical studies whereby "children with high individual salivary fluoride (0.075 ppm) were more frequently caries free ($p < 0.02$)" (Shields *et al.*, 1995; Shields *et al.*, 1997). More recently it has been reported that under continuously acidic conditions the optimum range of fluoride concentration able to reduce demineralisation was 0.1 – 135 ppm using scanning microradiography to capture demineralisation in real-time *in vitro* (Mohammed *et al.*, 2014).

In the present study, TMR revealed that lamination zones were present within the lesions of samples exposed to 0.05 ppm fluoride for 14 days, none in samples exposed for 4 days and the presence of a combination of laminated and non-laminated regions in day 10 samples. It is likely that this is due to the differential penetration of low level fluoride through the biofilm and into the depth of the caries lesion. It was previously shown, *in vitro*, that fluoride can penetrate up to 100 μm into sound enamel (Tyler and Poole, 1984) and partially into dentine when enamel is demineralised (ten Cate, 2001). This is likely due to the increased porosity of demineralised enamel, which has been described as more conducive to fluoride diffusion therefore protecting the underlying sound tissue (Lammers *et al.*, 1992). In the current study it can be proposed that as the enamel underwent the classic process

that leads to caries lesion formation, this low level of fluoride was insufficient to arrest demineralisation completely but as enamel porosity increased the few fluoride ions present in the current experimental design appear to have been able to penetrate to differential depths within the lesion body. Within the subsurface structure the formation of fluorapatite in localised zones would give rise to zones of lower solubility to subsequent acid diffusion. Furthermore at occasions of sucrose pulsing and subsequent acid production, fluorapatite crystals that dissolve buffer the solution less than hydroxyapatite (Brown *et al.*, 1977), meaning that the acids diffuse through the ever enlarging lesion pores with no neutralisation. This leads to the dissolution of the sound tissue beyond the original lesion and explains the formation of the further layers of demineralised tissue. However, the formation of fluoride enriched bands could also hinder the remineralisation of zones behind it by, in theory, denying the complete diffusion of fluoride deeper into the lesion. It has been shown elsewhere that acid susceptibility was increasingly higher in secondary portions of laminated lesions suggesting that less fluoride reached the second lesion than the first (Lagerweij and ten Cate, 2006). Laminated layers have been reported within natural caries lesions *in vivo* (Soni and Brudevold, 1959; Bergman and Lind, 1966; Silverstone, 1970); as well as in artificial caries *in vivo* (von der Fehr, 1967) and *in vitro* (Koulourides, 1977; Joyston-Bechal and Kidd, 1982; Kidd and Joyston-Bechal, 1982; ten Cate and Duijsters, 1983; Damato *et al.*, 1988; Arends *et al.*, 1992). Therefore, laminations are not simply a laboratory artefact (Lippert *et al.*, 2012). The presence of laminated layers is thought to reflect the history of fluoride administrations, indicating site-specificity in the efficacy of fluoride against the backdrop of patterns of fluctuations in de- and remineralisation (Silverstone, 1982; Schupbach *et al.*, 1990). It has been suggested that fluoride may be adsorbed at varying depths in varying concentrations throughout a laminated lesion and that this results in differences in the mineral susceptibility to acid (Driessens *et al.*, 1987; Lagerweij and ten Cate, 2006). Electron microscopy revealed that laminated zones

are less demineralised than the surrounding enamel in the body of a caries lesion and that there is greater evidence of resistance to demineralisation than the presence of remineralisation (Palamara *et al.*, 1986a). Within lamination zones in their study, Palamara *et al.* did not observe partial occlusion of voids by precipitation of new small crystals, needle-like crystals or amorphous material as reported for the remineralisation of the intact surface zone of incipient caries lesions (Palamara *et al.*, 1986b). Laminated zones have been purported as a transient phase in the arrest of the caries lesion (Driessens *et al.*, 1987) and they generally adhere to the formation of dissipative structures; regular spatial patterns arising in chemical systems far from equilibrium as a result of coupling between a chemical reaction and diffusion (Glansdorff and Prigogine, 1971). In these terms, during the formation of a carious lesion, the dissolution of apatite would constitute the primary chemical reaction alongside the diffusion of material in and out of the enamel. Since these laminated zones appeared only in the presence of fluoride ions, the current study emphasises the activity of fluoride in the caries process. The deeper lesions in the presence of 0.05 ppm fluoride agree with previous observations that fluoride aids in remineralising the original caries lesion but, in the process, drives the front of the lesion further into the tissue forming laminated structures (ten Cate *et al.*, 2006). The variations in lesion type between enamel samples and within sections of each sample is further testament to the heterogeneous nature of enamel caries developed within biological models. The tendency for laminations to occur more frequently at the interface between the biofilm exposed and nail-varnish protected areas of the enamel disc can be attributed to the accumulation of bacterial acids and fluoride at this interface and its subsequent combined effects on diffusion into the depths of the enamel.

5.4.3 Relationship between QLF and TMR in caries lesions with lamination zones

As discussed earlier (Section 3.4.2) the relationship between QLF ΔF and TMR ΔZ for the quantification of enamel caries is strong when the TMR technique has been undertaken successfully. Since the lesions within enamel exposed to a high frequency caries challenge and continuous 0.05 ppm fluoride contained lamination zones the relationship between these methods was investigated once again. The correlation between these variables paired for all individual replicate samples correlation was strong $r = 0.94$ ($p = 0.006$) and the existence of a proportional relationship between variables was evident ($p = 0.006$). The adjusted r^2 concluded that 85.1% of ΔF could be explained by ΔZ or vice-versa and it was confirmed that observations were independent. On this occasion the preparation of all sections for TMR was optimal. These findings once again support the necessity for optimal TMR procedures for reliable measures of enamel demineralisation to be obtained by this technique. This study also contributed further to the validation of QLF in accurately quantifying enamel caries. Nonetheless, the variation across the lesion body and between replicate samples highlights the potential advantage of QLF over TMR. Since TMR assesses single or multiple slices to provide a representation of the lesion on a whole whilst QLF measures the whole lesion non-destructively, comparison of the two methods is problematic. In this way *r* values obtained may inherently be modulated by the TMR process (ten Bosch, 2000). The heterogeneous nature of caries lesions formed by the complex multispecies biofilm and the difficulties in fully matching the areas assessed by both techniques could account for some of the disassociation quantified. Natural carious lesions can be highly variable in lesion depth and mineral loss and the slicing and subsequent grinding of enamel sections for TMR leads to some loss of lesion tissue. Furthermore, it would appear that the lamination zones may well influence the fluorescent ability of the caries lesion whilst QLF is able to

differentiate subtle differences in fluorescence change and therefore, the state of caries within a number of lesion types.

5.4.4 Enamel as a fluoride reservoir

It is understood that in the presence of solutions at concentrations above 100 ppm ionic fluoride, calcium fluoride forms. Saliva and the fluid phase of plaque become highly supersaturated with respect to calcium fluoride after application of topical fluoride agents (Vogel, 2011). At low pH, after the exposure of dental plaque biofilm to the sucrose challenge and subsequent production of acids by its microbial members, enamel dissolution would provide sufficient calcium for significant calcium fluoride formation. It has long been considered the primary source of bioavailable fluoride in the oral environment (Rolla and Saxegaard, 1990; Ogaard, 2001). There is evidence of this phenomenon in the findings of the present study. SEM of day 14 samples exposed to 1,450 ppm fluoride from day 4 onwards revealed calcium fluoride-like deposits of sizes ranging between 5.6 μm and 50.0 μm and EDX confirmed the presence of high levels of fluoride on the surface of these samples whilst proportions of calcium were similar to sound (unexposed/non-demineralised) enamel. The study confirms that fluoride was able to penetrate the biofilm and reach the surface of the underlying enamel. This agrees with previous reports that fluoride could penetrate biofilm when treated with 1,000 ppm fluoride for 30 minutes (Watson *et al.*, 2005). The dose-dependent penetration of fluoride into biofilms has also been suggested to be influenced by reductions in EPS thickness as concentrations of fluoride increased (Pandit *et al.*, 2013). The larger textured areas of joined mass may indicate areas of less net surface demineralisation, remineralisation or could be where globules merged. It has been shown that if loosely-bound fluoride covers the entire crystal surface it will not dissolve when pH falls as a consequence of bacterial acid production since its solubility would be similar to fluorapatite whereas if the coverage of loosely-bound fluoride is partial, the uncoated portions of the crystals dissolve (Arends and

Christoffersen, 1990). Fluoride was undetectable on the enamel surfaces after only 1 hour exposure to 1,450 ppm fluoride, suggesting that conditions and the factor of time were not sufficient for the deposition of significant quantities of fluoride for crystal growth or calcium fluoride formation. It was previously shown that calcium fluoride deposits from 905 ppm sodium fluoride topical treatment dissolved within an hour (Brudevold, 1974) and dissolution rates typically vary with complete dissolution within a day in exposed surfaces; to days and weeks in rough surfaces of caries lesions and in dental plaque (Brudevold, 1974; Lambrou *et al.*, 1981). Therefore, the current study suggests that prolonged exposure to a high concentration of fluoride facilitates the formation of these temporary fluoride stores. It would therefore, be interesting to investigate this phenomenon in enamel continuously exposed to lower concentrations of fluoride and at earlier times over the course of these experiments.

5.5 CONCLUSIONS

The model described in the present study facilitated the direct comparison of caries and biofilm derived from the same microbial inoculation process for the study of fluoride in caries control. The CDFF approach enabled the holistic investigation of both multispecies biofilm and enamel caries in response to low and high concentrations of fluoride. No other studies have employed the CDFF to study the effects of 0.05 ppm and 1,450 ppm fluoride in a biologically relevant caries model. Nonetheless, the present findings generally adhere to the current known effects of fluoride on the progression of caries in enamel as demonstrated in clinical, *in vivo*, *in situ* and *in vitro* studies.

In summary, the experiments of this study indicated that continuous exposure to 0.05 ppm fluoride did not diminish enamel demineralisation but lead to the formation of laminated zones within the resultant caries lesions by the tenth day. These

lamination zones were deemed a reflection of fluctuations in de- and remineralisation and the varied delivery of fluoride to the enamel beneath the biofilm of the system. It was also revealed that continuous exposure to 1,450 ppm sodium fluoride slurry exerted an anti-caries effect on enamel beneath multispecies biofilm. A small degree of surface erosion was observed but this was arrested over time. NCSP confirmed no statistically significant surface loss in enamel exposed to either concentration of fluoride. It was revealed that significantly high levels of fluoride persisted on enamel surfaces after exposure to 1,450 ppm fluoride from day 4 onwards of a 14 day model of caries compared to 1 hour exposure. SEM revealed calcium fluoride-like deposits of sizes ranging between 5.6 μm and 50.0 μm and EDX confirmed the presence of high levels of fluoride on the surface of these samples whilst proportions of calcium were similar to sound (unexposed/non-demineralised) enamel. Some areas also appeared resistant to demineralisation. The study confirms that fluoride was able to penetrate the biofilm and reach the surface of the underlying enamel. Therefore, the current study suggests that prolonged exposure to a high concentration of fluoride facilitates the formation of these temporary fluoride stores. It would therefore, be interesting to investigate this phenomenon in enamel continuously exposed to lower concentrations of fluoride and at earlier times over the course of these experiments. Continuous exposure to fluoride differentially influenced the microbial dynamic of mutans streptococci and *Lactobacillus* spp. within multispecies biofilm compared to non-sucrose exposed biofilm depending on its concentration. Fluoride possessed measurable antibacterial effects on total bacteria and the constituents of the biofilm compared to their proliferation in non-fluoride exposed biofilm. It would appear mutans streptococci persisted better in 0.05 ppm than in 1,450 ppm fluoride whilst *Lactobacillus* spp. persisted better in the presence of 1,450 ppm fluoride. In this way, the present study contributes to our understanding of the influence of habitation within multispecies biofilm on the metabolism and resistance to antimicrobials by caries associated bacteria. The present study provides evidence of ecological

adaptation in the finding that mutans streptococci were able to overcome the effects of 0.05 ppm fluoride and remained present, albeit at lower levels, in 1,450 ppm fluoride. This bacterial group was also resistant to the immediate reduction in viable cells imposed by 1,450 ppm fluoride after 1 hour, compared to other members of the microbial community. The pH of 0.05 ppm fluoride exposed biofilm was acidic and remained constant around critical pH, with no noticeable Stephan curve, in response to sucrose exposure. This can be attributed to the acid disposal and alkali generation capabilities of some members of the microbial community for survival as well as to the buffering capacity of hydroxyapatite as it is lost into the biofilm environment. It is also indicative of the absence of notable levels of *Lactobacillus* spp. in 0.05 ppm fluoride since this bacterial type are known to rapidly produce lactic acid during homo-fermentation as well as alcohols via hetero-fermentation and typically thrive in acidic conditions. Furthermore, it has previously been shown that the optimal concentration of fluoride to effect EPS formation in *S. mutans* biofilm was 100 ppm (Pandit *et al.*, 2013); therefore it is likely that EPS formation by the mutans streptococci exposed to low level fluoride in the current study was not altered. Early limitations in the quantities of mutans streptococci are attributable to the heightened formation of EPS under conditions of higher sucrose availability and the degradation of these insoluble polysaccharides between each sucrose pulse would lead to further and prolonged periods of biofilm acidification. Increased EPS formation has been directly associated with increased enamel demineralisation in previous studies. Therefore, the quantity of specific bacterial species in a given oral biofilm cannot be a primary indicator of caries risk. The behaviour of biofilm community members in response to environmental stresses is the most important factor in ascertaining their specific role in the cariogenicity of biofilm.

Finally, the relationship between TMR and QLF in caries lesions containing lamination zones was excellent providing further evidence of the suitability of QLF in quantifying caries *in vitro*. These findings once again support the necessity for optimal TMR procedures for reliable measures of enamel demineralisation to be obtained by this technique. Nonetheless, there was further evidence of the heterogeneous nature of caries lesions formed by the complex multispecies biofilm suggesting a potential advantage of QLF over TMR. Since TMR assesses single or multiple slices with loss of lesion tissue to provide a representation of the lesion on a whole whilst QLF measures the whole lesion non-destructively, comparison of the two methods is problematic. Furthermore, it would appear that the lamination zones may well influence the fluorescent ability of the caries lesion whilst QLF is able to differentiate subtle differences in fluorescence change and therefore, the state of caries within a number of lesion types.

Chapter 6 Modelling the effects of frequency of fluoride delivery in a biological caries model

6.1 INTRODUCTION

There is strong scientific evidence that the daily use of fluoride containing toothpaste is effective in preventing caries in permanent teeth and this is primarily a result of maintaining elevated levels of fluoride in the mouth throughout the course of a day (Twetman *et al.*, 2003; Twetman, 2009; Walsh *et al.*, 2010). Several studies reveal that the frequency of brushing with fluoride toothpastes is significantly associated with caries prevalence and that brushing twice daily or more exerts a greater preventative effect than once daily (Marinho *et al.*, 2003). It was found that irregular behaviour in evening tooth brushing was highly associated with high caries prevalence (Julihn *et al.*, 2006) and caries was 20 – 30% higher in subjects who brushed only once a day compared to those who brushed twice (Chestnutt *et al.*, 1998; Ashley *et al.*, 1999). Additionally, as the duration of fluoride exposure increases, it appears that its penetration into dental plaque increases (Watson *et al.*, 2005) but little is known about the effect of frequency of exposure in this regard. There is a lack of studies evaluating whether a third daily use of fluoride dentifrice confers additional anti-caries benefit. One *in vivo* study investigated the retention of fluoride in subject plaque and saliva as well as its influence on plaque pH when using high fluoride (5000 ppm F^-) or standard toothpaste (1450 ppm F^-) twice or thrice a day (Nordström and Birkhed, 2013). To date, no other studies have modelled the effect of a third daily exposure of fluoride on the microbial ecology of multispecies biofilm and on caries in enamel. The CDFF approach developed in the current work is an appropriate framework to test this hypothesis.

Aims

This study aimed to investigate the impact on caries of thrice daily exposure to fluoride solution compared with twice daily exposure within a biologically relevant biofilm exposed to a high frequency sucrose challenge. The retention of fluoride as a function of concentration, biofilm age and frequency of fluoride exposure was also modelled.

6.2 Experimental Procedure

6.2.1 Effect of twice-daily compared to thrice-daily fluoride delivery in a novel dual CDFF caries – biofilm model

Two 8-pan CDFFs containing no sample pans were sterilised by dry heat (Section 2.5). Sixteen sample pans were loaded with a combination of 4.8 mm HA (Clarkson Chromatography Products, South Williamsport, PA, USA) and 5 mm bovine enamel discs (Intertek 4-Front, Chester, UK) recessed to 200 μm. Each pan contained three enamel discs, half-painted with nail varnish, and two hydroxyapatite discs (Section 2.4). The sample pans were sterilised by gamma irradiation (Section 2.5) and introduced aseptically into the turntable of each CDFF so that the position of each pan and its contents were comparable between CDFFs. Biofilm was produced in both CDFFs simultaneously by inoculating in the same way; connected to the same saliva growth medium inoculated with one aliquot of saliva pool. These methods are fully described in Chapter 2 and in the experimental procedure of Chapter 4 (Section 4.2) and 5 (Section 5.2).

Ten litres of artificial saliva growth media was prepared in one vessel as described in Section 2.6. 5 mL artificial saliva growth media was aseptically removed from the vessel in a laminar flow environment and 5 mL of a sterile 100 ppm fluoride standard (Orion Thermo, Orion Instruments, MA, USA) was added to the vessel so that the

final concentration of fluoride was 0.05 ppm. 10 hours after inoculation began, flow from the inoculation flask was stopped and the sterile artificial saliva growth media with added fluoride was delivered at 0.267 mL min^{-1} into both CDFFs over the course of the experiment as the replenishing media supply.

Ten litres of 110 mM sucrose solution (Sigma-Aldrich, Poole, UK) was prepared and autoclaved to sterilise before being connected aseptically to both 8-pan CDFFs. As in Chapter 5, the sucrose solution was pulsed at a flow rate of 2.7 mL min^{-1} via a timer controlled peristaltic pump every 2 hours 8 times daily for 15 mins with a 10 hour rest period each day. The final concentration of sucrose, taking into account the dilution effect of the artificial saliva growth media being pumped in concurrently, was expected to be 100 mM. Again, sucrose flow was first initiated 8 hours after the flow of sterile artificial saliva growth media began.

A solution of sodium fluoride (AnalR, BDH, Poole, England, UK) was prepared, so that the final concentration was 1,450 ppm fluoride and sterilised. From day 4 of the experiment, the sodium fluoride solution was delivered into the CDFFs at a flow rate of 5 mL min^{-1} either two times or three times daily using a timer-controlled peristaltic pump via the side-arm of a growback trap in the tubing line feeding sterile artificial saliva growth medium (Figure 6.1). In a separate experiment, a sterile solution of sodium fluoride with a final concentration of 228 ppm fluoride was prepared and delivered into the CDFFs either two times or three times daily in the same manner.

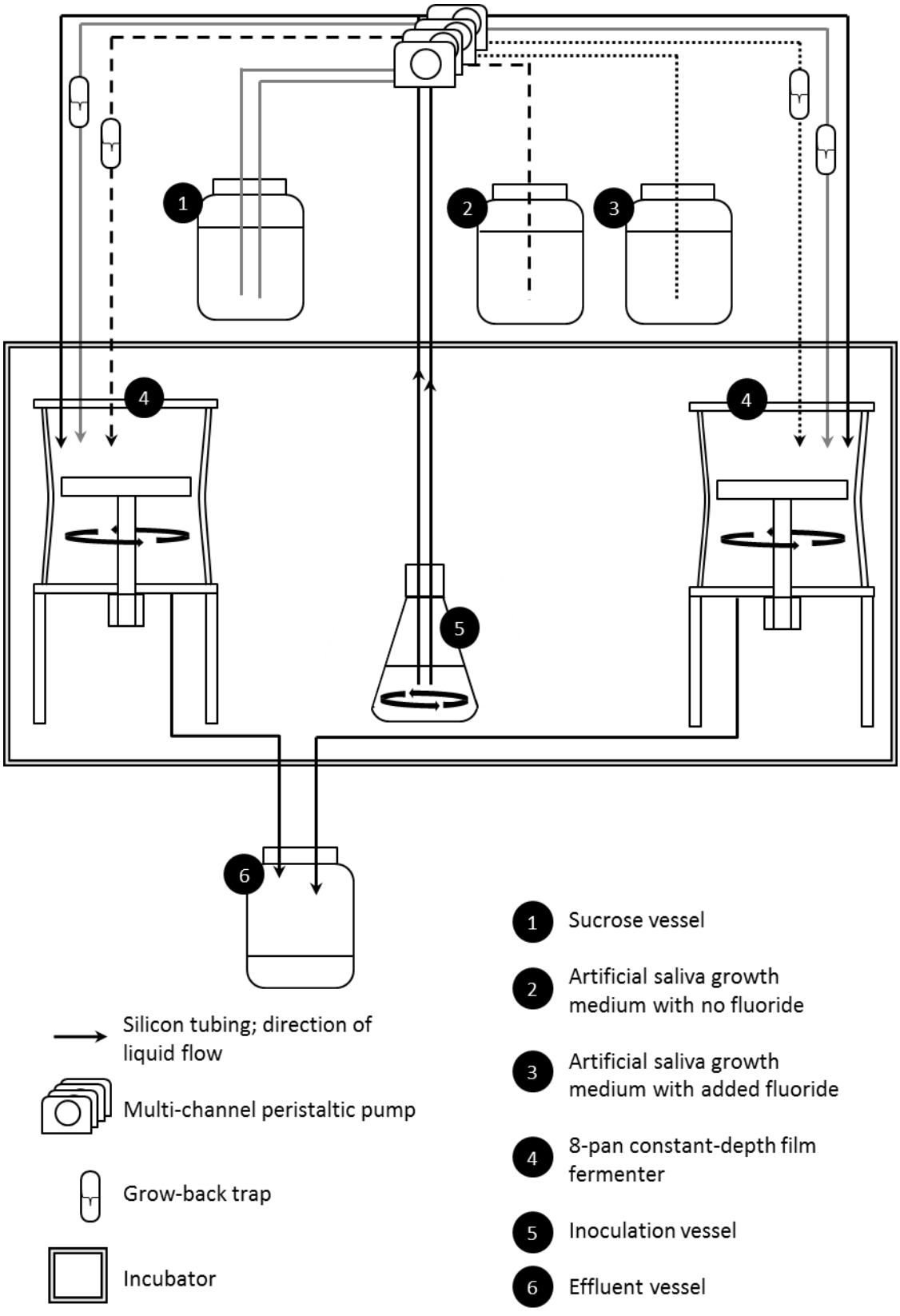


Figure 6.1. Circuit diagram with key of experimental procedure to investigate the effect of sucrose concentration within a constant-depth film fermenter caries-biofilm model.

In both experiments samples were taken on day 4 (immediately before fluoride flow began), day 5, 8, 10, 12 and 14. Biofilm from these sample pans was analysed to isolate and enumerate total viable bacteria, total *Streptococcus* spp, total mutans streptococci group and *Lactobacillus* spp. using selective solid agar as described in section 3.5.1. Enamel discs half painted with nail varnish were photographed with QLF and analysed for fluorescence loss (see Section 2.6.1.), scanned for surface changes using NCSP (see Section 2.6.2.) and finally subjected to TMR methodology to elucidate any sub-surface changes in mineral content as described in section 2.6.4. In the 1,450 ppm fluoride exposed experiment additional enamel samples were extracted on day 6 and day 15, whereas in the 228 ppm fluoride exposed experiment additional samples were extracted at day 6 whilst no enamel samples were extracted at day 15.

6.2.2 Investigating the retention of fluoride in a dual CDFF microcosm caries model

The retention of fluoride within the model system was investigated in three ways. First abiotically; wherein one 8-pan CDFF was fully loaded with sample pans with all plugs recessed to 200 μm. Deionised water was pumped into the CDFF at 0.267 mL min^{-1}, commensurate with the flow rate used to deliver artificial saliva in experiments involving the 8-pan CDFF. A solution of sodium fluoride was prepared so that the final concentration was 1450 ppm fluoride and pumped into the CDFF at 5 mL min^{-1} for 2 mins via the sidearm of the growback trap. Samples were intercepted at the effluent port before sodium fluoride flow was initiated (to capture a baseline measure) and at 1, 1.5, 2, 5, 10, 15, 20, 30 minutes, 1, 1.5, 2, 3, 4, 5, 6, 7, 23 and 24 hours after sodium flow began. A sample taken directly from the sodium fluoride reservoir provided a positive control measure.

To investigate fluoride clearance after biofilm exposure to fluoride, samplings from the effluent were taken on day 7 and 13 of the dual CDFF experiment exposed to 1450 ppm fluoride; and on day 13 of the 228 ppm fluoride exposed experiment. During the dual CDFF experiments described in section 6.2.1 samples were intercepted at the effluent ports of both CDFFs before sodium fluoride flow (baseline) and 0.5, 1.5, 2.5, 3.5, 4.5, and 5.5 hours after sodium fluoride flow began. All samples were measured for fluoride concentration using a fluoride probe as described in Section 2.7.3. Additionally, biofilm of the 1,450 ppm exposed model system was sampled on day 15 and the plaque fluoride measured (Section 2.7.3).

6.2.3 Statistical analyses

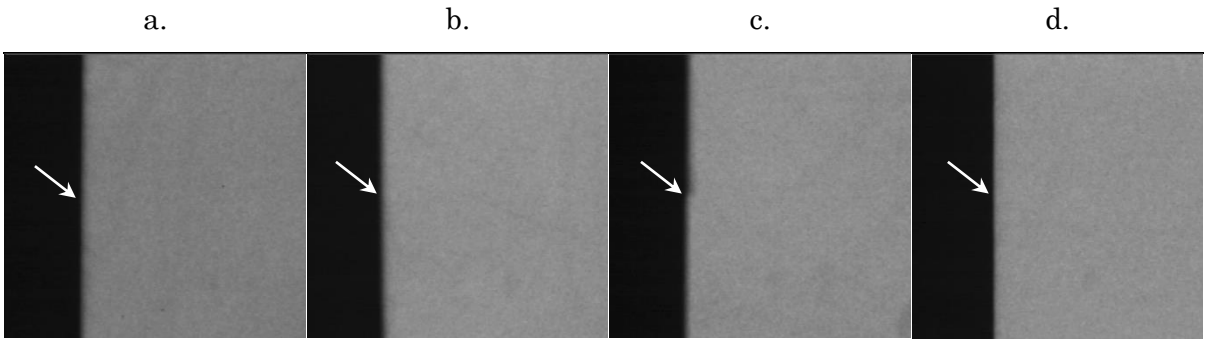
The hypothesis that there are differences in enamel mineral content (as assessed by QLF and TMR) and in surface changes (quantified by NCSP) when exposed to microcosm biofilm subjected to caries challenge together with 1,450 ppm fluoride or 228 ppm fluoride twice or thrice daily was tested using single factor ANOVA and two-way ANOVA analyses. Sidak's multiple comparisons tests were used post-hoc to identify the statistical significance of differences between outcomes (a) at each time within a given experiment and (b) for differences between each experiment for each time point. An analysis of covariance (ANCOVA) model able to adjust for time was used to test the hypothesis that changing frequency of fluoride exposure and concentration of fluoride affects the growth of microbial constituents of the biofilm. Prior to all analysis normal distributions of the raw data was checked and transformed where needed and all datasets were routinely checked for outliers as described in Section 2.9.

6.3 RESULTS

6.3.1 Effect of twice versus thrice daily fluoride exposure and concentration on enamel demineralisation

TMR revealed that no subsurface caries lesions were present in enamel beneath biofilm exposed to a high frequency sucrose caries challenge and either 1,450 ppm fluoride or 228 ppm fluoride both twice and thrice daily (Figure 6.2). By day 14 mineral loss (ΔZ, %Vol.μm) was 275.00 ± 42.43 SD (n = 2) and 300.00 ± 28.28 SD (n = 2) in enamel exposed to 1,450 ppm fluoride twice and thrice daily respectively (Figure 6.3b). Whilst in enamel exposed twice and thrice daily to 228 ppm fluoride ΔZ was 356.25 ± 30.05 SD (n = 2) and 347.50 ± 3.536 SD (n = 2) respectively (Figure 6.4b). ANCOVA revealed that differences in ΔZ were statistically significant according to fluoride exposure ($p < 0.001$) when comparing between frequencies in exposure and differences in fluoride concentration; whilst time was significant only when comparing 228 ppm fluoride exposed either twice or thrice daily ($p = 0.009$). However, estimated marginal means analysis revealed that no comparisons were statistically significant according to mineral loss once the influence of time was corrected for (Table 6.1). Subsequent multiple comparisons testing showed that differences in ΔZ over time were not statistically significant except for between day 4 and 5 samples in samples exposed to 1,450 ppm fluoride three times daily (mean difference = 290.00, $p = 0.029$).

Figure 6.2. Example TMR images of sections of enamel exposed to cariogenic biofilm pulsed with 100 mM sucrose 8 times daily over 14 days and 1,450 ppm fluoride (a) twice or (b) thrice daily or 228 ppm fluoride (c) twice or (d) thrice daily. The interface between the biofilm exposed and nail-varnish protected areas of the enamel disc (indicated by arrow).



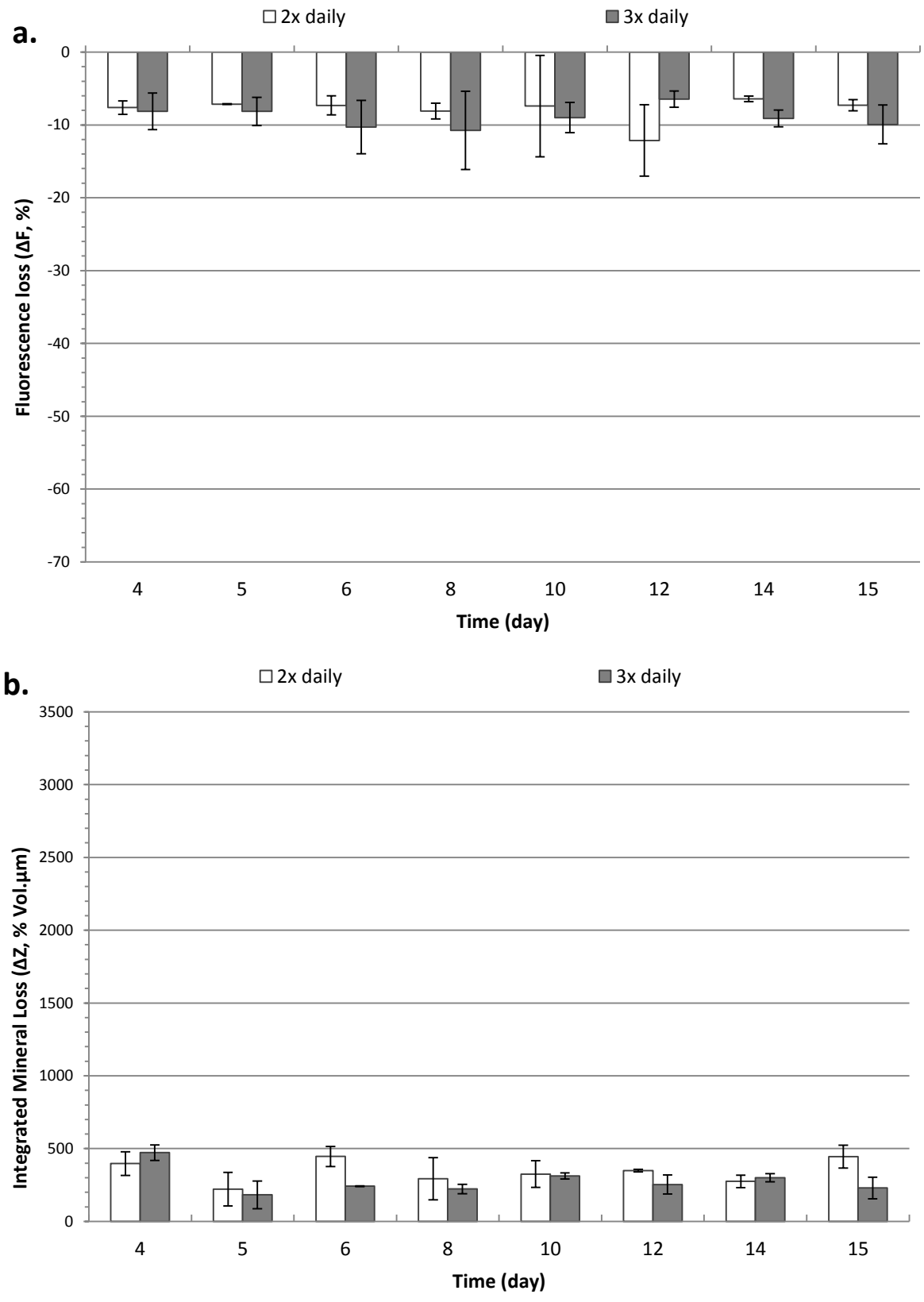


Figure 6.3. Longitudinal changes in (a) enamel fluorescence (average fluorescence loss, ΔF, %) as detected by QLF *in vitro* and (b) mineral loss (average ΔZ, %Vol.µm) determined by TMR in bovine enamel samples exposed to multispecies oral biofilm pulsed with 100 mM sucrose 8 times daily and **1,450 ppm fluoride** either twice (white bars) or thrice (grey bars) daily. Error bars represent standard deviation, n = 3.

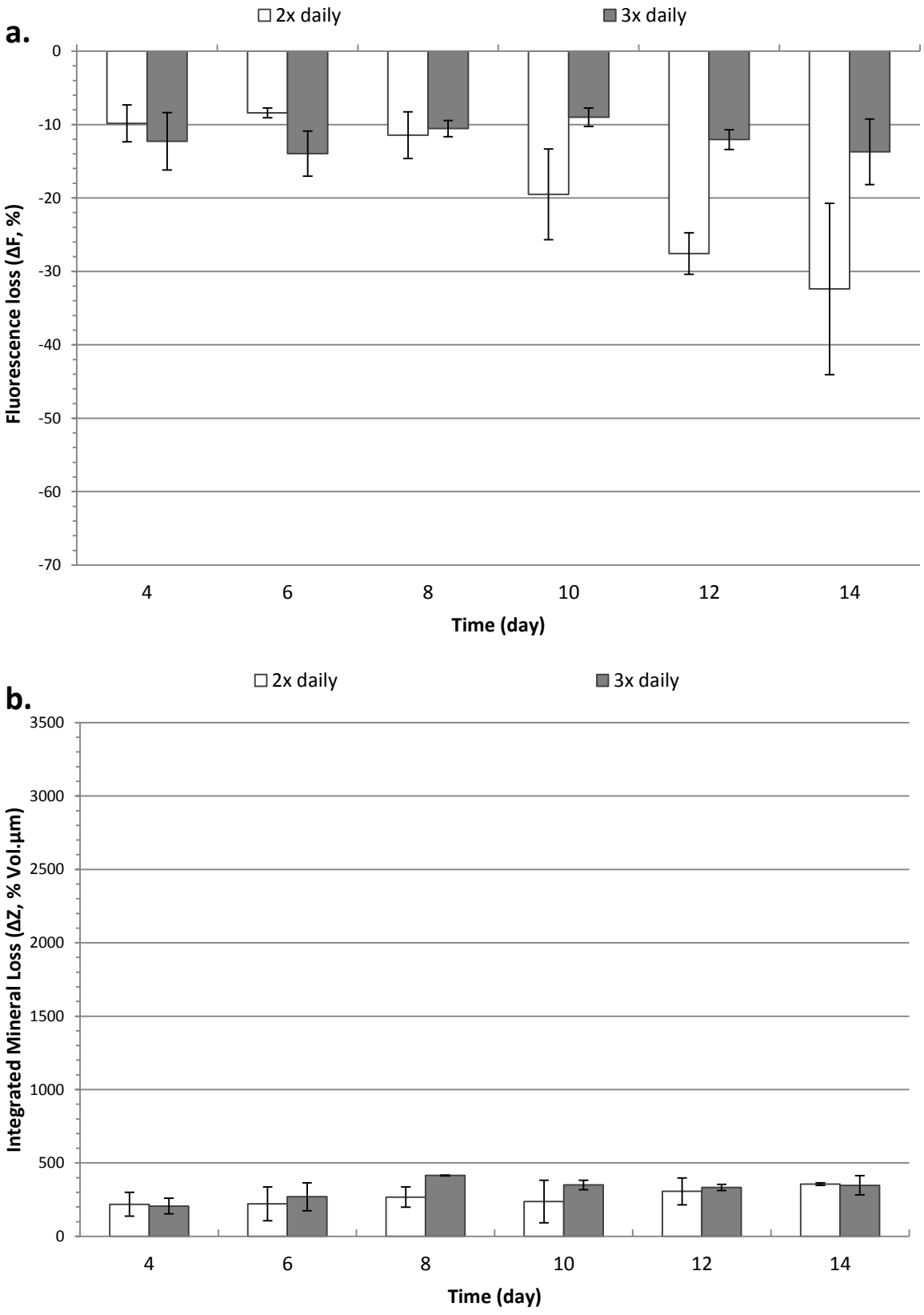


Figure 6.4. Longitudinal changes in (a) enamel fluorescence (average fluorescence loss, ΔF, %) as detected by QLF *in vitro* and (b) mineral loss (average ΔZ, %Vol.µm) determined by TMR in bovine enamel samples exposed to multispecies oral biofilm pulsed with 100 mM sucrose 8 times daily and **228 ppm fluoride** either twice (white bars) or thrice (grey bars) daily. Error bars represent standard deviation, n = 3.

Table 6.1. Results of statistical testing examining the effect of frequency of fluoride addition or concentration on fluorescence change and mineral loss based on linearly independent pairwise comparisons among estimated marginal means.

Fluoride exposure comparisons	Fluorescence change (ΔF)	Mineral Loss (ΔZ)
1,450 ppm 2x daily versus 3x daily	F = (1,31) 0.000 $p = 0.983$	F = (1,20) 0.225 $p = 0.640$
228 ppm 2x daily versus 3x daily	F = (1,32) 16.674 **$p < 0.001$**	F = (1,20) 3.113 $p = 0.093$
1,450 ppm versus 228 ppm 2x daily	F = (1,31) 37.002 **$p < 0.001$**	F = (1,20) 1.057 $p = 0.234$
1,450 ppm versus 228 ppm 3x daily	F = (1,32) 11.682 **$p = 0.002$**	F = (1,20) 0.633 $p = 0.436$

F-statistic; F. *$\log_{10}$ transformed prior to analysis.
bold font denotes difference is statistically significant.

ΔF observed in samples exposed to 1,450 ppm fluoride both twice or thrice daily was deemed not clinically significant as well as for those exposed to 228 ppm fluoride three times daily (Figure 6.3a and Figure 6.4a). By day 14 fluorescence change (ΔF, %) was -6.42 ± 0.39 SD ($n = 3$) and -9.11 ± 1.14 SD ($n = 3$) in samples exposed to 1,450 ppm fluoride twice and thrice daily respectively. However, QLF distinguished substantial ΔF in enamel exposed to 228 ppm twice daily compared to those exposed thrice daily to the same concentration (Figure 6.4a and Figure 6.5c & d respectively). ΔF was -13.72 ± 4.47 SD ($n = 3$) in samples exposed to 228 ppm fluoride three times daily compared to -32.39 ± 11.68 ($n = 3$) in samples exposed twice daily. ANCOVA revealed that differences in ΔF were statistically significant according to fluoride exposure ($p < 0.001$ for 1,450 ppm 2x vs 3x, and 3x 1,450 vs 3x 228 ppm; $p = 0.001$ for 228 ppm 2x vs 3x and $p = 0.034$ for 2x 1,450 vs 2x 228 ppm fluoride) when comparing between frequencies in exposure and differences in fluoride concentration whilst time was significant only when comparing 228 ppm fluoride exposed either twice or thrice daily ($p < 0.001$) and when comparing concentrations exposed twice daily ($p < 0.001$). However, estimated marginal means analysis revealed that differences in ΔF when

comparing samples exposed to 1,450 ppm fluoride either twice or thrice daily were not statistically significant (Table 6.1). Meanwhile differences between 228 ppm fluoride samples exposed twice or thrice daily were statistically significant ($p < 0.001$) as well as when comparing concentrations either exposed twice ($p < 0.001$) or three times daily ($p = 0.002$; Table 6.1). Subsequent multiple comparisons testing showed that differences in ΔF over time were not statistically significant except for in samples exposed to 228 ppm fluoride twice daily (Table 6.2). When comparing ΔF of day 4 and 5 samples with those from days 12 and 14 differences were statistically significant, whilst day 8 was also found to differ from day 14 samples statistically significantly suggesting the progression of mineral loss in twice daily 228 ppm fluoride exposed enamel.

Figure 6.5. Typical QLF-D images of bovine enamel sampled longitudinally within a biological caries model subjected to 100 mM sucrose 8 times daily and 1,450 ppm fluoride (a) twice daily or (b) thrice daily; or 228 ppm fluoride (c) twice daily or (d) thrice daily.

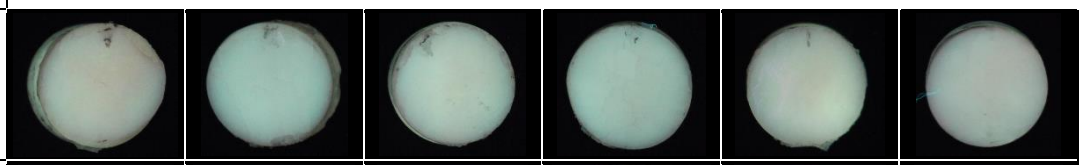
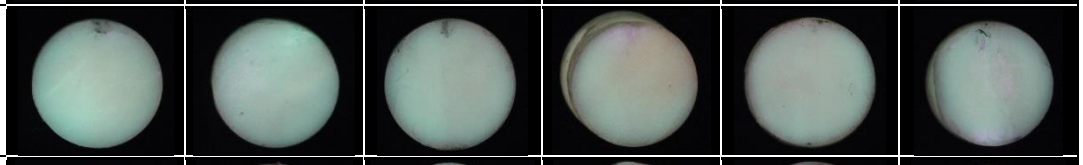
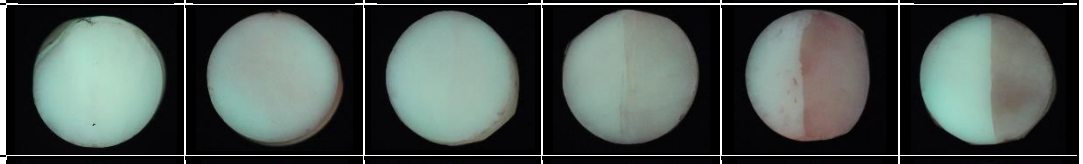
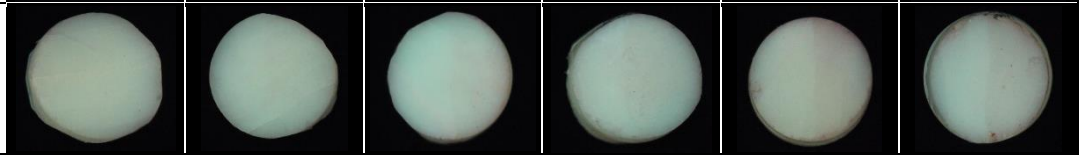
ID	Time (day)					
	4	6	8	10	12	14
a.						
b.						
c.						
d.						

Table 6.2. Results of multiple comparisons testing the statistical significance between changes in enamel caries characteristics over 14 days in a caries model exposed to 100 mM sucrose 8 times daily and **228 ppm fluoride twice daily** according to fluorescence change, mineral loss, lesion depth and surface loss.

Time (day)	Fluorescence change (ΔF, %)			Mineral Loss *(ΔZ, %Vol.μm)*			Surface Loss* (μm)		
	MD	±CI$^{95\%}$	*p*	MD	±CI$^{95\%}$	*p*	MD	±CI$^{95\%}$	*p*
4 vs 12	17.7	0.617, 34.8	**0.040**	-87.5	-443, 267	0.909	.00498	-0.00504, 0.0150	0.779
4 vs 14	22.6	5.45, 39.7	**0.007**	-137.5	-493, 218	0.657	.00103	-0.0090, 0.0111	1.00
5 vs 12	19.2	2.05, 36.3	**0.023**	-85.0	-440, 270	0.918	.00700	-0.0030, 0.0170	0.326
5 vs 14	24.0	6.88, 41.1	**0.004**	-135.0	-490, 220	0.671	.00305	-0.0070, 0.0131	0.994
8 vs 14	21.0	3.85, 38.07	**0.012**	-88.8	-444, 267	0.905	.00594	-0.00408, 0.0160	0.552

Mean difference, MD; upper and lower confidence intervals, ±CI$^{95\%}$

*natural $\log_{10}$ transformed prior to analysis; **bold** font denotes difference is statistically significant

6.3.2 Effect on enamel surface loss

In general, no surface loss was detected in enamel beneath biofilm exposed to the sucrose challenge and 1,450 ppm fluoride thrice daily throughout the course of the experiment (Figure 6.6). Maximum surface loss was observed in enamel samples pulsed with the caries challenge and 1,450 ppm fluoride twice daily by 12 days (40 nm ± 83 nm) with no detected surface loss in preceding days. Surface loss at day 14 was lower (17 nm ± 20 nm) and no surface loss was observed by day 15. Differences in surface loss were not statistically significant according to frequency of fluoride exposure ($p = 0.228$, $F(2,32) = 1.548$, $n = 18$), time ($p = 0.132$, $F(1,32) = 2.385$, $n = 18$) and their interaction ($p = 0.176$, $F(1,32) = 1.918$, $n = 18$). Analysis of estimated marginal means also showed no statistical significance in the effect of fluoride frequency when time was discounted from the statistical model ($p = 0.322$, $F(1,32) = 1.013$, $n = 18$). For enamel exposed to 228 ppm fluoride either twice or thrice daily, no surface loss was observed at days 4 and 6. Some surface loss was observed by the eighth and tenth days regardless of fluoride exposure and differences were found not

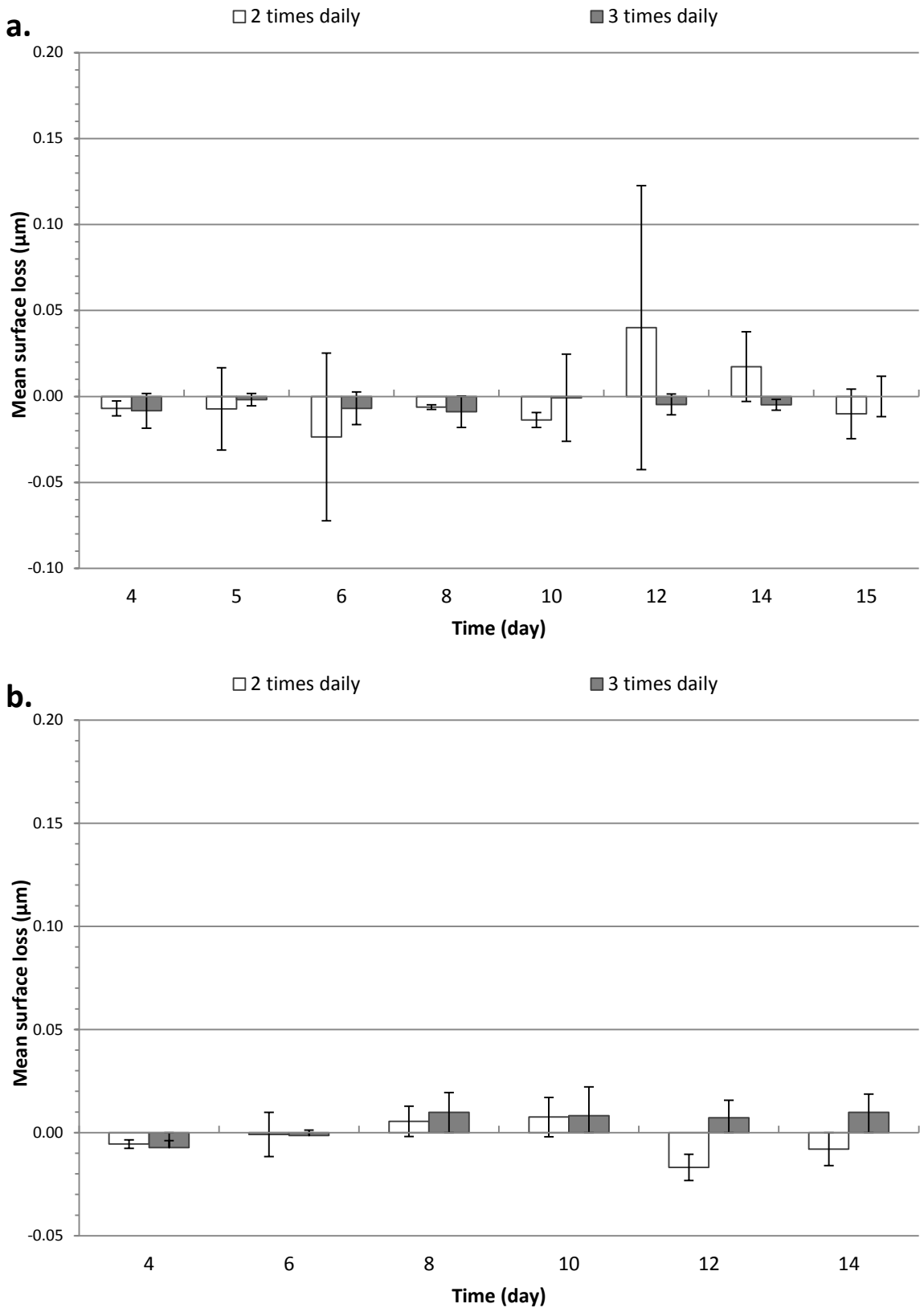


Figure 6.6. Mean surface loss (µm) of bovine enamel samples exposed to 100 mM sucrose 8 times daily and either (a) 1,450 ppm or (b) 228 ppm sodium fluoride twice or thrice daily as detected by NCSP measurements of height differences between negative control and treatment zones within samples. Error bars represent standard deviation, n = 3.

to be statistically significant. However, in day 12 and 14 samples exposed thrice daily, surface loss was observed whilst none was detected in those exposed twice daily. Once again, differences were found not to be statistically significant according to frequency of fluoride exposure ($p = 0.295$, $F(2,32) = 1.270$, $n = 18$) and time ($p = 0.434$, $F(1,32) = 0.628$, $n = 18$). The interaction between fluoride exposure and time was found to be statistically significant ($p = 0.016$, $F(1,32) = 6.511$, $n = 18$) as well as the effect of fluoride frequency when the factor of time was removed ($p = 0.025$, $F(1,32) = 5.523$, $n = 18$). Furthermore, analysis of estimated marginal means when comparing samples exposed to either 228 ppm or 1,450 ppm fluoride three times daily revealed that differences in surface loss were statistically significant ($p = 0.007$, $F(1,32) = 8.370$, $n = 18$).

6.3.3 Biofilm formation and growth in the presence of 1,450 ppm or 228 ppm fluoride twice or thrice daily

Figure 6.7 shows the microbial composition of multispecies oral biofilm when exposed to 1,450 ppm fluoride (a) twice or (b) thrice daily along with 100 mM sucrose 8 times daily. The same is presented in Figure 6.8 except demonstrating bacterial growth when pulsed with 228 ppm fluoride (a) twice or (b) thrice daily. Viable counts revealed that pseudo-steady state, in terms of total viable bacteria, was achieved in all biofilm by day 4 though a degree of exponential increase continued in biofilm exposed to 1,450 ppm fluoride twice daily ($6.15 \times 10^4 \pm 2.51 \times 10^4$ SD cfu mm^{-2} at day 4 compared to $8.01 \times 10^7 \pm 1.92 \times 10^7$ SD cfu mm^{-2}, $n = 4$ at day 14). Prior to exposure to fluoride (day 4) bacterial counts from each CDFF of the model were similar according to the viable of mutans streptococci and *Lactobacillus* spp. Results indicate that mutans streptococci were able to proliferate according to similar trends when comparing exposures to 1,450 ppm fluoride two times or three times daily. Yet *Lactobacillus* spp. appear to continue proliferation to the end of the experiment in twice daily exposed biofilm compared with the steady state achieved in thrice daily

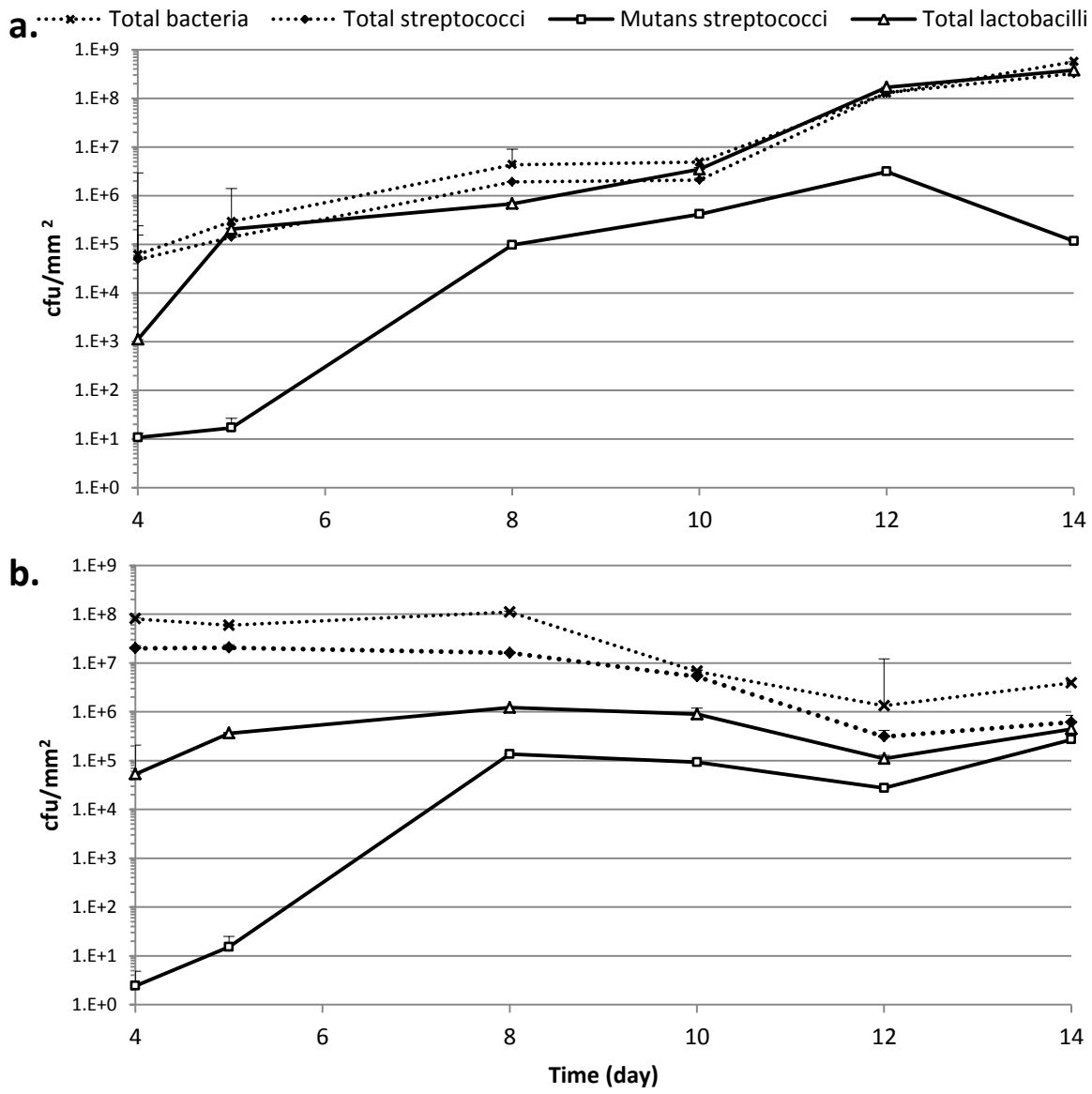


Figure 6.7. Microbial composition of multispecies oral biofilm grown on hydroxyapatite pulsed with 100 mM sucrose eight times daily and exposed to 1,450 ppm fluoride either (a) twice or (b) thrice daily. Error bars represent standard deviation, $n = 4$.

exposed biofilm. Pseudo-steady state was achieved by day 8 in mutans streptococci during both treatments with continuously higher levels of *Lactobacillus* spp. than mutans streptococci throughout the experiment irrespective of frequency of fluoride exposure. Mutans streptococci reached $9.38 \times 10^5 \pm 1.46 \times 10^6$ SD cfu mm^{-2} ($n = 4$) and $1.31 \times 10^5 \pm 1.02 \times 10^5$ SD cfu mm^{-2} ($n = 4$) in twice and thrice daily exposures respectively (average of day 8 – 14 counts, $n = 4$). Whereas *Lactobacillus* spp. reached $1.37 \times 10^8 \pm 1.78 \times 10^8$ SD cfu mm^{-2} ($n = 4$) when exposed twice daily and $6.65 \times 10^5 \pm 4.88 \times 10^5$ SD cfu mm^{-2} (average of day 8 – 14 counts, $n = 4$) when exposed thrice daily.

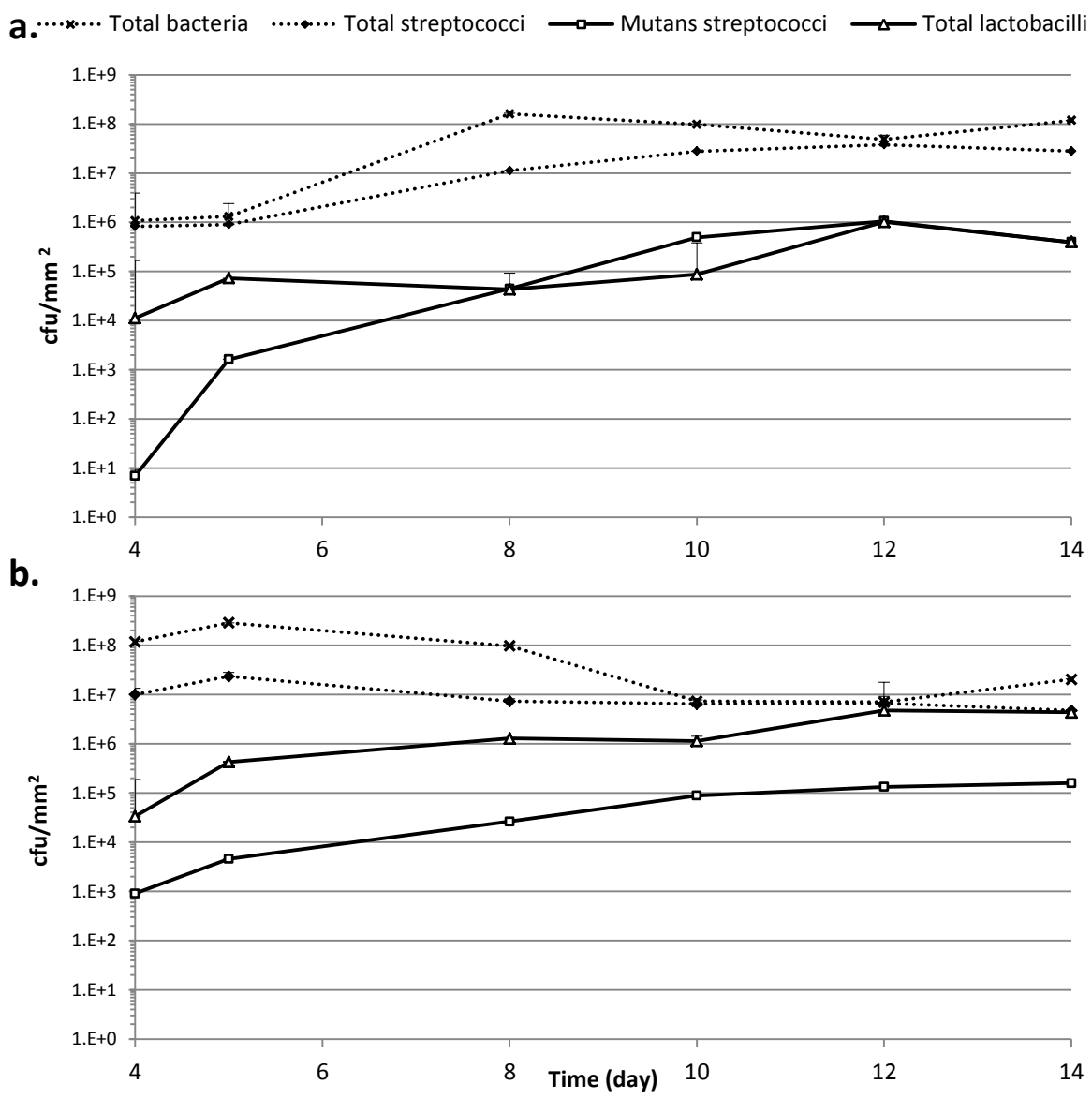


Figure 6.8. Microbial composition of multispecies oral biofilm grown on hydroxyapatite pulsed with 100 mM sucrose eight times daily and exposed to 228 ppm fluoride either (a) twice or (b) thrice daily. Error bars represent standard deviation, n = 4.

Statistical testing revealed that differences over time were statistically significant in all microbial groups (Tables 6.3 – 6.6) and that frequency of fluoride exposure as well as its interaction with time was statistically significant for total bacteria, *Streptococcus* spp. and *Lactobacillus* spp. but not for mutans streptococci. In biofilm exposed twice or thrice daily with 228 ppm fluoride, pseudo-steady state was achieved by both mutans streptococci and *Lactobacillus* spp. by day 8 (Figure 6.8). Mutans streptococci reached levels similar to those observed in 1,450 ppm fluoride exposed biofilm for both twice daily ($4.96 \times 10^5 \pm 4.18 \times 10^5$ SD cfu mm^{-2} (n = 4) and thrice daily

$1.02 \times 10^5 \pm 5.81 \times 10^4$ SD cfu mm^{-2} (n = 4) exposures of 228 ppm fluoride (average of day 8 – 14 counts, n = 4). *Lactobacillus* spp. were present at lower levels when exposed to 228 ppm fluoride twice daily ($3.85 \times 10^5 \pm 4.48 \times 10^5$ SD cfu mm^{-2} (average of day 8 – 14 counts, n = 4) than those observed in 1,450 ppm fluoride pulsed twice daily. Whereas *Lactobacillus* spp reached $2.88 \times 10^6 \pm 1.94 \times 10^6$ SD cfu mm^{-2} (average of day 8 – 14 counts, n = 4) when exposed thrice daily to 228 ppm fluoride; higher than observed in biofilm exposed to 1,450 ppm fluoride thrice daily. When comparing biofilm exposed twice and thrice daily to 228 ppm fluoride, statistical testing revealed that differences over time were statistically significant in *Streptococcus* spp. and *Lactobacillus* spp. and mutans streptococci but not in total bacteria (Tables 6.7 – 6.10). Differences according to frequency of fluoride exposure were statistically significant for all microbial groups while the interaction of fluoride exposure and time was statistically significant in total bacteria, *Streptococcus* spp. and mutans streptococci but not in *Lactobacillus* spp.

Statistical testing of the effect of concentration when biofilm was exposed to fluoride twice daily with either 1,450 ppm or 228 ppm fluoride revealed that differences in total bacteria, *Streptococcus* spp. and *Lactobacillus* spp. were statistically significant whilst differences in mutans streptococci were not (Tables 6.11 – 6.14). Differences over time were statistically significant for all microbial groups while the interaction between fluoride concentration and time was statistically significant in total bacteria, *Streptococcus* spp. and *Lactobacillus* spp. but not in mutans streptococci. When testing the effect of concentration on biofilm exposed to fluoride three times daily, statistical testing revealed that differences were statistically significant for all microbial groups according to concentration as well as for time (Tables 6.15 – 6.18). The interaction between these factors was statistically significant for *Streptococcus* spp., mutans streptococci and *Lactobacillus* spp. but not for total bacteria.

Table 6.3. Results of ANCOVA testing statistical significance of time and fluoride addition in **total viable bacteria*** exposed to caries challenge and **1,450 ppm fluoride twice or thrice daily.**

Source of Variation	SSIII	*df*	MS	F	*p*
Fluoride	296.903	2	148.451	1225.062	**< 0.001**
Time	6.532	1	6.532	53.900	**< 0.001**
Fluoride x time	47.145	1	47.145	389.050	**< 0.001**

R Squared = 0.998 (Adjusted R Squared = 0.998)

Table 6.4. Results of ANCOVA testing statistical significance of time and fluoride addition in **total *Streptococcus* spp.*** exposed to caries challenge and **1,450 ppm fluoride twice or thrice daily.**

Source of Variation	SSIII	*df*	MS	F	*p*
Fluoride	264.629	2	132.314	1137.964	**< 0.001**
Time	5.949	1	5.949	51.167	**< 0.001**
Fluoride x time	49.711	1	49.711	427.541	**< 0.001**

R Squared = 0.998 (Adjusted R Squared = 0.997)

Table 6.5. Results of ANCOVA testing statistical significance of time and fluoride addition in **mutans streptococci*** exposed to caries challenge and **1,450 ppm fluoride twice or thrice daily.**

Source of Variation	SSIII	*df*	MS	F	*p*
Fluoride	1.544	2	0.711	0.613	0.546
Time	141.983	1	141.983	112.815	**< 0.001**
Fluoride x time	0.375	1	0.375	0.298	0.588

R Squared = 0.941 (Adjusted R Squared = 0.935)

Table 6.6. Results of ANCOVA testing statistical significance of time and fluoride addition in **total *Lactobacillus* spp.*** exposed to caries challenge and **1,450 ppm fluoride twice or thrice daily.**

Source of Variation	SSIII	*df*	MS	F	*p*
Fluoride	102.541	2	51.270	172.805	**< 0.001**
Time	43.136	1	43.139	145.400	**< 0.001**
Fluoride x time	32.644	1	32.644	110.025	**< 0.001**

R Squared = 0.993 (Adjusted R Squared = 0.992)

Type III sum of squares, SSIII; degrees of freedom, *df*; mean square, MS; F = (MS between groups)/(MS within groups). *$\log_{10}$ transformed prior to analysis. **bold** font denotes difference is statistically significant.

Table 6.7. Results of ANCOVA testing statistical significance of time and fluoride addition in **total viable bacteria*** exposed to caries challenge and **228 ppm fluoride twice or thrice daily.**

Source of Variation	SSIII	*df*	MS	F	*p*
Fluoride	366.449	2	183.225	756.191	**< 0.001**
Time	0.652	1	0.652	2.692	0.108
Fluoride x time	18.385	1	18.385	75.876	**< 0.001**

R Squared = 0.996 (Adjusted R Squared = 0.996)

Table 6.8. Results of ANCOVA testing statistical significance of time and fluoride addition in **total *Streptococcus* spp.*** exposed to caries challenge and **228 ppm fluoride twice or thrice daily.**

Source of Variation	SSIII	*df*	MS	F	*p*
Fluoride	278.692	2	139.346	1919.553	**< 0.001**
Time	2.429	1	2.429	33.467	**< 0.001**
Fluoride x time	7.550	1	7.550	104.007	**< 0.001**

R Squared = 0.999 (Adjusted R Squared = 0.998)

Table 6.9. Results of ANCOVA testing statistical significance of time and fluoride addition in **mutans streptococci*** exposed to caries challenge and **228 ppm fluoride twice or thrice daily.**

Source of Variation	SSIII	*df*	MS	F	*p*
Fluoride	20.057	2	10.029	21.041	**< 0.001**
Time	69.663	1	69.663	146.158	**< 0.001**
Fluoride x time	8.245	1	8.245	17.299	**< 0.001**

R Squared = 0.979 (Adjusted R Squared = 0.977)

Table 6.10. Results of ANCOVA testing statistical significance of time and fluoride addition in **total *Lactobacillus* spp.*** exposed to caries challenge and **228 ppm fluoride twice or thrice daily.**

Source of Variation	SSIII	*df*	MS	F	*p*
Fluoride	110.187	2	55.093	387.160	**< 0.001**
Time	16.244	1	16.244	114.152	**< 0.001**
Fluoride x time	0.102	1	0.102	0.714	0.403

R Squared = 0.996 (Adjusted R Squared = 0.995)

Type III sum of squares, SSIII; degrees of freedom, *df*; mean square, MS; F = (MS between groups)/(MS within groups). *$\log_{10}$ transformed prior to analysis. **bold** font denotes difference is statistically significant.

Table 6.11. Results of ANCOVA testing statistical significance of time and fluoride addition in **total viable bacteria*** exposed to caries challenge and **1,450 ppm or 228 ppm fluoride twice daily.**

Source of Variation	SSIII	*df*	MS	F	*p*
Fluoride	141.843	2	70.921	352.117	**< 0.001**
Time	52.688	1	52.688	261.590	**< 0.001**
Fluoride x time	4.680	1	4.680	23.234	**< 0.001**

R Squared = 0.996 (Adjusted R Squared = 0.996)

Table 6.12. Results of ANCOVA testing statistical significance of time and fluoride addition in **total *Streptococcus* spp.*** exposed to caries challenge and **1,450 ppm or 228 ppm fluoride twice daily.**

Source of Variation	SSIII	*df*	MS	F	*p*
Fluoride	129.489	2	64.745	573.734	**< 0.001**
Time	47.584	1	47.584	421.662	**< 0.001**
Fluoride x time	6.717	1	6.717	59.520	**< 0.001**

R Squared = 0.998 (Adjusted R Squared = 0.998)

Table 6.13. Results of ANCOVA testing statistical significance of time and fluoride addition in **mutans streptococci*** exposed to caries challenge and **1,450 ppm or 228 ppm fluoride twice daily.**

Source of Variation	SSIII	*df*	MS	F	*p*
Fluoride	1.174	2	0.587	0.540	0.586
Time	144.152	1	144.152	132.627	**< 0.001**
Fluoride x time	0.622	1	0.622	0.572	0.454

R Squared = 0.954 (Adjusted R Squared = 0.950)

Table 6.14. Results of ANCOVA testing statistical significance of time and fluoride addition in **total *Lactobacillus* spp.*** exposed to caries challenge and **1,450 ppm or 228 ppm fluoride twice daily.**

Source of Variation	SSIII	*df*	MS	F	*p*
Fluoride	57.895	2	28.948	122.475	**< 0.001**
Time	63.946	1	63.946	270.550	**< 0.001**
Fluoride x time	18.361	1	18.361	77.683	**< 0.001**

R Squared = 0.994 (Adjusted R Squared = 0.993)

Type III sum of squares, SSIII; degrees of freedom, *df*; mean square, MS; F = (MS between groups)/(MS within groups). *$\log_{10}$ transformed prior to analysis. **bold** font denotes difference is statistically significant.

Table 6.15. Results of ANCOVA testing statistical significance of time and fluoride addition in **total viable bacteria*** exposed to caries challenge and **1,450 ppm or 228 ppm fluoride three times daily.**

Source of Variation	SSIII	*df*	MS	F	*p*
Fluoride	521.509	2	260.755	1608.963	**< 0.001**
Time	15.173	1	15.173	93.625	**< 0.001**
Fluoride x time	0.172	1	0.172	1.064	0.308

R Squared = 0.997 (Adjusted R Squared = 0.997)

Table 6.16. Results of ANCOVA testing statistical significance of time and fluoride addition in **total *Streptococcus* spp.*** exposed to caries challenge and **1,450 ppm or 228 ppm fluoride three times daily.**

Source of Variation	SSIII	*df*	MS	F	*p*
Fluoride	413.832	2	206.916	2721.935	**< 0.001**
Time	8.412	1	8.412	110.654	**< 0.001**
Fluoride x time	2.928	1	2.928	38.520	**< 0.001**

R Squared = 0.998 (Adjusted R Squared = 0.998)

Table 6.17. Results of ANCOVA testing statistical significance of time and fluoride addition in **mutans streptococci*** exposed to caries challenge and **1,450 ppm or 228 ppm fluoride three times daily.**

Source of Variation	SSIII	*df*	MS	F	*p*
Fluoride	20.427	2	10.213	16.107	**< 0.001**
Time	69.425	1	69.425	109.488	**< 0.001**
Fluoride x time	8.829	1	8.829	13.924	**0.001**

R Squared = 0.969 (Adjusted R Squared = 0.966)

Table 6.18. Results of ANCOVA testing statistical significance of time and fluoride addition in **total *Lactobacillus* spp.*** exposed to caries challenge and **1,450 ppm or 228 ppm fluoride three times daily.**

Source of Variation	SSIII	*df*	MS	F	*p*
Fluoride	154.832	2	77.416	382.034	**< 0.001**
Time	6.770	1	6.770	33.407	**< 0.001**
Fluoride x time	3.053	1	3.053	15.065	**< 0.001**

R Squared = 0.994 (Adjusted R Squared = 0.994)

Type III sum of squares, SSIII; degrees of freedom, *df*; mean square, MS; F = (MS between groups)/(MS within groups). *$\log_{10}$ transformed prior to analysis. **bold** font denotes difference is statistically significant.

When adjusting for time as a covariate estimated marginal means analysis revealed the statistical significance of differences in bacterial counts in response to the effect of fluoride frequency or concentration alone (Table 6.19). Differences in *Lactobacillus* spp. were statistically significant both when frequency of exposure and when fluoride concentration differed. Total bacteria differed significantly when fluoride concentration was differed but not when testing differences in response to an increase of frequency in 228 ppm fluoride exposure. Counts of *Streptococcus* spp. differed significantly in response to changes in fluoride concentration when exposed both twice and thrice daily but differences when comparing the effect of frequency at both fluoride concentrations were not statistically significant. Lastly, differences in mutans streptococci were significant only according to the effect of fluoride concentration when biofilm was exposed thrice daily. Differences between twice and thrice daily exposures were not statistically significant either in 1,450 ppm or 228 ppm exposed fluoride and when comparing viable counts in response to fluoride concentration exposed twice daily.

Table 6.19. Results of statistical testing examining the effect of frequency of fluoride addition or concentration on constituents of multispecies oral biofilm based on linearly independent pairwise comparisons among estimated marginal means.

	Bacterial group			
Fluoride exposure comparisons	Total bacteria*	*Streptococcus* spp.*	Mutans streptococci*	*Lactobacillus* spp.*
1,450 ppm 2x versus 3x	F = (1,44) 21.907 ***p* < 0.001**	F = (1,44) 1.593 *p* = 0.214	F = (1,43) 1.777 *p* = 0.190	F = (1,44) 24.159 ***p* < 0.001**
228 ppm 2x versus 3x	F = (1,44) 2.813 *p* = 0.101	F = (1,44) 0.291 *p* = 0.592	F = (1,44) 0.072 *p* = 0.789	F = (1,44) 67.875 ***p* < 0.001**
1,450 versus 228 ppm 2x	F = (1,44) 22.070 ***p* < 0.001**	F = (1,44) 14.358 ***p* < 0.001**	F = (1,44) 0.695 *p* = 0.409	F = (1,44) 78.563 ***p* < 0.001**
1,450 versus 228 ppm 3x	F = (1,44) 10.501 ***p* = 0.002**	F = (1,44) 12.840 ***p* = 0.001**	F = (1,43) 9.347 ***p* = 0.004**	F = (1,44) 10.751 ***p* = 0.002**

F-statistic; F. *$\log_{10}$ transformed prior to analysis.

bold font emphasises difference is statistically significant.

6.3.4 Fluoride retention within biofilm after twice or thrice daily exposure to 228 or 1,450 ppm fluoride

Results revealed that fluoride is retained within the CDFFs of this model with and without biofilm present (Figure 6.9). In biofilm exposed to 1,450 ppm fluoride for 7 days higher concentrations of fluoride were observed in effluent samples from the CDFF exposed thrice daily than in those exposed two times daily 5.5 hours after fluoride exposure (1.006 ppm vs 0.665 ppm respectively). This trend was consistent throughout the retention experiment. Similarly, in biofilm exposed to 228 ppm fluoride for 13 days effluent fluoride concentrations were higher in the thrice daily exposed (0.132 ppm) compared to the twice daily exposed CDFF (0.045 ppm) 5.5 hours post fluoride exposure and this was also consistent throughout. However, in biofilm exposed for 13 days to 1,450 ppm fluoride levels of fluoride were higher in effluent samples from the CDFF pulsed with fluoride twice daily compared to those from the thrice daily exposed CDFF. 5.5 hours after fluoride exposure 1.766 ppm fluoride was observed in effluent from twice daily exposed biofilm compared to 1.069 ppm fluoride in biofilm exposed thrice daily. Nevertheless, 1 hour post fluoride exposure, fluoride in effluent samples from thrice daily exposed biofilm was 419.417 ppm compared to 292.470 ppm in twice daily exposed biofilm effluent. Yet fluoride was consistently higher in twice daily exposed biofilm at subsequent times and until the end of the experiment. Furthermore, direct analysis of the fluoride in day 15 biofilm revealed 0.179 ppm and 0.244 ppm fluoride in plaque exposed to 1,450 ppm fluoride twice and thrice daily respectively. All differences when comparing CDFFs within each dual CDFF model were not statistically significant when tested with ANCOVA adjusting for the influence of time. Finally, it appears that between sucrose pulses fluoride concentrations remain relatively stable and the largest decreases in fluoride concentration coincide with sucrose pulse events.

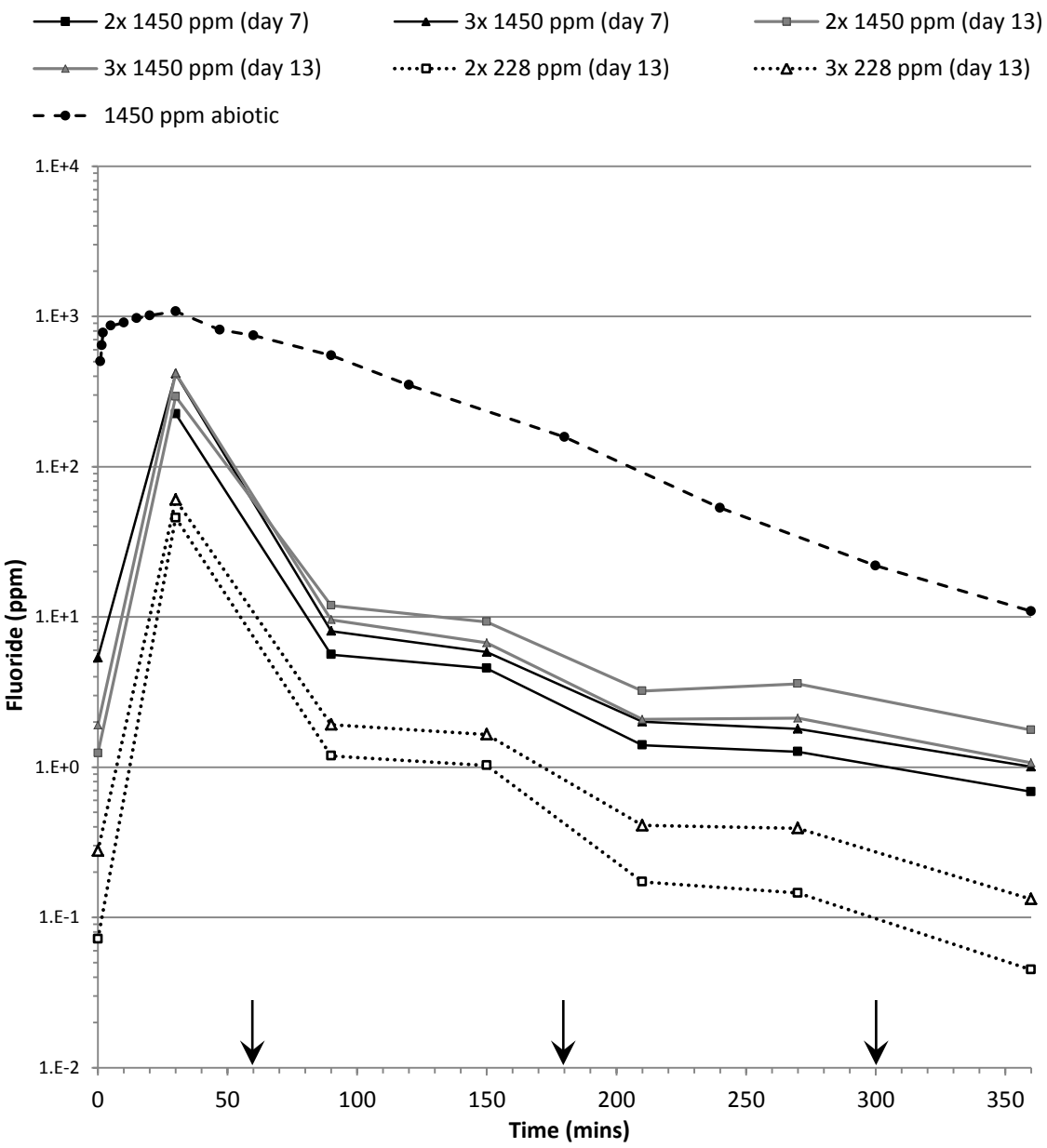


Figure 6.9. Concentrations of fluoride (ppm) retained within CDFFs operated with biofilm exposed to 1,450 ppm or 228 ppm fluoride twice or thrice daily after 7 and 13 days compared to a CDFF operated abiotically and exposed to 1,450 ppm fluoride. Arrows indicate occasions during the timeline when sucrose was pulsed into CDFFs containing biofilm.

6.4. DISCUSSION

A number of clinical studies support the concept that the amount of fluoride in oral reservoirs is crucial in its anti-caries effect, since an inverse relationship has been demonstrated between caries incidence and the concentration of fluoride in saliva and/or dental plaque (Gaugler and Bruton, 1982; Schamschula *et al.*, 1985; Nobre dos Santos *et al.*, 2002). Maintaining a cariostatic concentration of fluoride in oral fluids is vitally important in increasing the anti-caries effects of fluoride agents (ten Cate, 1997; Featherstone, 2000). This is chiefly achieved by the release of the ion from bioavailable reservoirs on the teeth, oral mucosa and dental plaque that can persistently increase fluoride concentrations in the fluids in contact with de- and remineralising teeth. The latter is particularly interesting due to the association of dental plaque with the caries disease process and could be manipulated to provide an aide in achieving anti-caries effects. Plaque fluid fluoride concentrations were significantly higher than corresponding saliva fluoride concentrations prior to and 30 and 60 minutes after rinsing with a sodium fluoride solution (Vogel *et al.*, 1992a). Saliva fluoride concentrations were initially higher relative to baseline than corresponding plaque fluoride values in studies of whole plaque fluoride (Zero *et al.*, 1992). However, it appeared that the rate of fluoride clearance was slower in plaque than the rate observed in saliva (Zero *et al.*, 1992). This likely reflects the binding of fluoride to specific plaque reservoir sites (Rose *et al.*, 1996), though it may also involve the restricted diffusion of fluoride ions within the plaque matrix (McNee *et al.*, 1982; Watson *et al.*, 2005). Considering that most individuals cannot completely remove dental plaque after toothbrushing, the amount of fluoride retained in plaque can help determine the fate of the enamel beneath it (Pessan *et al.*, 2006; Pessan *et al.*, 2008; Pessan *et al.*, 2010; Buzalaf *et al.*, 2011). Since fluoride retained in plaque can be released under acidic conditions during cariogenic challenges, its retention so close to the site of de- and remineralisation is highly clinically relevant. In other words, it

means that fluoride is potentially released and made available when it is needed, to reduce demineralisation or to enhance remineralisation of early lesions.

Standard widely available sodium fluoride containing toothpaste is formulated at no more than 1,500 ppm or1,150 ppm in the EU and USA respectively (Lippert, 2013) and recently launched prescription fluoride toothpastes containing 2,800 and 5,000 ppm fluoride have been shown to significantly lower caries progression compared to 1,100 – 1,450 ppm fluoride toothpastes (Baysan *et al.*, 2001; Ekstrand *et al.*, 2008; Nordström and Birkhed, 2010). During standard use, toothpastes slurry with saliva and disperse active ingredients, leading to a successive decrease in fluoride concentration over time. 228 ppm fluoride is close to the maximum in saliva-dentifrice slurry samples retrieved 30 seconds after a 1 minute brushing period with toothpastes containing 1,000 or 1,500 ppm fluoride (Bruun *et al.*, 1987). It is also similar to the concentration of fluoride found in current widely available fluoride rinse formulations (Vogel *et al.*, 2000). Salivary fluoride concentration tended to increase as the concentration of fluoride in the applied fluoride product increased (Bruun *et al.*, 1984) and a linear relationship between log fluoride and log time was reported by both groups of authors. Therefore, salivary fluoride clearance generally adheres to a typical curve with an initial phase of rapid loss followed by a secondary phase of slower clearance (Duckworth and Morgan, 1991). Here, it was elucidated that *in vivo* the initial phase lasted from 40 – 80 minutes depending on the individual. This was likely influenced by user-related factors such as technique and post-product rinsing with water. Nonetheless, it appears the quantity of fluoride in saliva soon after brushing is proportional to the original concentration of fluoride applied. In effect, the current study models the efficacy of 5,000 ppm and 1,450 ppm products once diluted by saliva into 1,450 ppm and 228 ppm fluoride respectively. However, it also models the effect of 1,450 ppm and 228 ppm fluoride as the maximum applied and the

instance whereby products of these concentrations were not diluted by the effects of saliva, spitting, rinsing and swallowing.

6.4.1 Retention of fluoride within a CDFF biofilm model

Results of the present study generally agree with *in vivo* findings reported previously (Nordström and Birkhed, 2013). The outcomes indicate that fluoride is retained within the CDFFs of the model with and without biofilm present. A slower rate of clearance was observed in the abiotic model, likely a consequence of the absence of oral biofilm occupying the volume of the recessed plugs within each pan, the absence of enamel capable of fluoride uptake and the lack of intermittent pulses of sucrose solution. As in the previous study (Nordström and Birkhed, 2013), the retention of fluoride in biofilm by day 15 after exposure to 1,450 ppm fluoride from day 4 onwards, was greater when biofilm was pulsed thrice daily compared to twice daily (0.244 ppm vs 0.179 ppm fluoride). Higher levels of fluoride were detected in artificial saliva effluent samples of biofilm exposed thrice daily than in those exposed twice daily to 1,450 ppm fluoride by the seventh day (1.006 ppm vs 0.665 ppm respectively) and to 228 ppm fluoride by the thirteenth day (0.132 ppm vs 0.045 ppm).

However, by the thirteenth day the concentration of fluoride in effluent samples from the CDFF pulsed thrice daily with 1,450 ppm fluoride was lower than in effluent from the twice daily pulsed CDFF 5.5 hours after exposure to fluoride (1.069 ppm vs 1.766 ppm). Nonetheless, the rate of clearance did not appear to be faster overall and the level of fluoride in thrice daily pulsed was similar to concentrations observed at day 7 exposed to the same frequency. The concentration of fluoride in effluent was higher in the effluent of thrice daily exposed biofilm prior to the first sucrose challenge. On a whole, it appears that between sucrose pulses fluoride concentrations remain relatively stable and the largest decreases in fluoride concentration coincide with

sucrose pulse events. In the case of 1,450 ppm fluoride less fluoride was present in the effluent of thrice daily exposed biofilm than in the effluent of twice daily exposed biofilm after the first sucrose pulse event and until the end of the experiment.

6.4.2 Growth of bacterial members of multispecies biofilm under caries challenge and twice or thrice daily exposure to 228 ppm or 1,450 ppm and its effects on enamel demineralisation

The findings of the present study reveal that the growth of total bacteria, *Streptococcus* spp., mutans streptococci and *Lactobacillus* spp. all significantly differed when pulsed with either 228 ppm or 1,450 ppm fluoride thrice daily. All groups also significantly changed when biofilm was exposed to 228 ppm fluoride either twice daily or thrice daily. Differences in viable counts for total bacteria, *Streptococcus* spp. and *Lactobacillus* spp. were statistically significant when biofilm was exposed to 1,450 ppm fluoride twice or thrice daily but differences for mutans streptococci were not. The same was observed when inspecting the viable counts for biofilm supplied with either 1,450 ppm or 228 ppm fluoride twice daily.

The results indicate that increasing the frequency of fluoride delivery did not yield a significant antibacterial effect on mutans streptococci exposed to 1,450 ppm fluoride yet it did on *Lactobacillus* spp. within the same multispecies biofilm. Viable counts of *Lactobacillus* spp. were significantly lower in thrice than in twice daily exposed biofilm ($6.65 \times 10^5 \pm 4.88 \times 10^5$ SD cfu mm^{-2} vs $1.37 \times 10^8 \pm 1.78 \times 10^8$ SD cfu mm^{-2} respectively). It appears that this bacterial group continued exponential proliferation to the end of the experiment in twice daily exposed biofilm compared with the steady state achieved in thrice daily exposed biofilm whereas, there was no statistical basis for differences in mutans streptococci in biofilm exposed to 1,450 ppm fluoride either twice or thrice daily. There were statistically significant differences over time but not in the interaction between fluoride frequency and time. Therefore, it is likely mutans

streptococci were able to proliferate according to similar trends regardless of frequency of exposure to 1,450 ppm fluoride. On the other hand, viable counts of mutans streptococci were significantly lower at steady state when exposed to 228 ppm fluoride three times daily compared to twice daily ($1.02 \times 10^5 \pm 5.81 \times 10^4$ SD cfu mm^{-2} vs $4.96 \times 10^5 \pm 4.18 \times 10^5$ SD cfu mm^{-2} respectively). However, pulsing with 228 ppm fluoride thrice daily resulted in significantly higher bacterial counts of *Lactobacillus* spp. at steady state than when biofilm was exposed twice daily ($2.88 \times 10^6 \pm 1.94 \times 10^6$ SD cfu mm^{-2} vs $3.85 \times 10^5 \pm 4.48 \times 10^5$ SD cfu mm^{-2} respectively). This indicates that at this concentration the additional exposure time did not reduce the persistence of this bacterial group within the biofilm community.

Fluoride concentration alone did not appear to exert a significant antibacterial effect on mutans streptococci when exposed twice daily but a significant difference was observed in biofilm exposed three times daily to 228 ppm or to 1,450 ppm fluoride. This is supported by analysis of the estimated marginal means which standardises time by comparing between measures at the mid-time-point within the course of the experiment. However, quantities of this bacterial group at steady state in biofilm exposed thrice daily to 1,450 ppm fluoride were, in fact, higher than in biofilm pulsed with 228 ppm fluoride thrice daily ($1.31 \times 10^5 \pm 1.02 \times 10^5$ SD cfu mm^{-2} vs $1.02 \times 10^5 \pm 5.81 \times 10^4$ SD cfu mm^{-2} respectively). Additionally, quantities of *Lactobacillus* spp. at steady state were also consistently higher in 1,450 ppm fluoride than in 228 ppm fluoride regardless of whether biofilm was pulsed twice or three times and these differences were statistically significant. Nonetheless, since this an assessment between sequential CDFF experiments one must be cautious in the interpretation of this comparison. The variability and difference between biofilm of CDFFs was significantly reduced within the dual model compared to when CDFFs are run on separate occasions from different aliquots of saliva pool (see Chapter 4). Therefore, it is better to compare between CDFFs within the dual model system than sequentially,

particularly when subtle effects are expected. In this regard, the current study would benefit from additional dual CDFF experiments with one exposed to 228 ppm fluoride and the other to 1,450 ppm fluoride as an ideal for comparing concentrations applied at each frequency and to substantiate the findings of the present study.

As discussed earlier, evidence indicates that the fluoride sensitivity of various genera and species is markedly varied and influenced by the acidic conditions of the plaque environment. It has been shown that the sensitivity of bacteria to fluoride greatly increased as pH fell (Shiota, 1956; Jenkins, 1959). It was previously shown (see Chapter 5) that mutans streptococci were most supressed under continuous exposure to 1,450 ppm fluoride along with the sucrose challenge, providing evidence that at this concentration and method of delivery fluoride exerted major additive antimicrobial effects on this bacterial group. This was not achieved in the current study, suggesting that when 1,450 ppm fluoride is delivered to biofilm twice or thrice throughout the day, it cannot effectively exert major bactericidal effects. Overall, the addition of a third application of fluoride had no detectable effect on this bacterial group. In the previous study (see Chapter 5) counts of *Lactobacillus* spp. were slightly lower in the presence of 1,450 ppm fluoride continuously but this difference was statistically significant. In the present study, it would appear the intermittent and third pulse of 1,450 ppm fluoride supressed the exponential growth of this bacterial group. In the current study fluoride was delivered for two minutes, 1 hour before the first sucrose exposure, 1 hour after the fourth sucrose exposure and 1 hour after the last sucrose pulse. The results for the retention of fluoride in the model indicate that pulsing with sucrose was an additive factor in the clearance of fluoride from the CDFF system. By the time a new pulse of fluoride was to be introduced the effluent fluoride concentration was around 1 ppm in 1,450 ppm fluoride exposures and below 0.2 ppm fluoride when exposed with 228 ppm fluoride. It generally appeared that fluoride was

present at higher levels for longer when either concentration was delivered three times in a day compared to twice.

Predominant opinion is that the effect of fluoride on enamel solubility and the promotion of remineralisation is its principal mode in achieving cariostatic and anti-caries effects (Geddes and Bowen, 1990) and that its multitude of effects on bacterial cells is likely less important (van der Hoeven and Franken, 1984). The results of the current study support this since caries associated bacteria were not overwhelmingly inhibited in the presence of fluoride at biologically representative frequencies and concentrations. Samples of both CDFFs in each dual experiment were fed with 0.05 ppm fluoridated artificial saliva growth medium continuously to represent the background levels of fluoride in saliva reported previously (Bruun *et al.*, 1984; Zero *et al.*, 1992). TMR revealed that no subsurface caries lesions were present in enamel beneath biofilm exposed to a high frequency sucrose caries challenge and either 1,450 ppm fluoride or 228 ppm fluoride both twice and thrice daily. Statistical testing confirmed that mineral loss did not progress over 14 days regardless of the frequency or concentration of sodium fluoride exposed. QLF generally supported the TMR observations except for samples exposed to 228 ppm fluoride twice daily wherein some substantial fluorescence loss was observed over the time course. Time was a statistically significant factor only when comparing samples exposed to 228 ppm fluoride twice or thrice daily and when comparing the two concentrations exposed twice daily. This suggests that changes in fluorescence differed significantly when samples are exposed twice daily to 228 ppm compared to 1450 ppm fluoride and when the frequency of exposure to 228 ppm fluoride is increased. This is supported by the estimated marginal means analysis which also quantified statistically significant differences between fluorescence changes in enamel exposed to either concentration three times daily when the factor of time was standardised.

6.5 CONCLUSIONS

The CDFF model developed in the studies of this thesis was capable of modelling the retention of fluoride and findings were consistent with those from *in vivo* studies. The present study confirmed the anti-caries ability of 1,450 ppm and 228 ppm fluoride solutions delivered both twice and thrice daily for 10 days. It would appear that fluoride primarily exerted its beneficial effects by directly modulating enamel demineralisation. Increasing the frequency of fluoride application differentially affected the microbial constituents of the multispecies biofilm and quantities of specific caries associated bacteria did not appear to directly relate to the lack of significant enamel demineralisation at these concentrations. Nonetheless, the QLF detection of some fluorescence loss and red fluorescence at the enamel surface of samples exposed to 228 ppm twice daily indicates the beneficial effect of a third daily exposure to fluoride at this concentration. When pulsed thrice daily with 1,450 ppm higher levels of fluoride were retained in biofilm and would likely be more caries protective in the long-term.

Chapter 7 Investigating biofilm fluorescence with QLF-D™ and multispectral imaging

7.1 INTRODUCTION

It has been shown that biomolecules fluoresce at different wavelengths hence they exhibit different colour profiles, due mainly to the structure and composition of intrinsic fluorophores including; red fluorescent porphyrins (Konig *et al.*, 1994); UV fluorescent tryptophan and tyrosine (Creed, 1984b, a) and green fluorescent NADH, NAPH and flavins (Salmon *et al.*, 1982; Galland and Senger, 1988). A wide range of oral bacteria exhibit red and green fluorescence in different proportions so that they appear red, green or orange in colour when illuminated with the 405 nm wavelength light used in QLF technology (Lennon *et al.*, 2006; Volgenant *et al.*, 2013). The detection of endogenous fluorophores can yield information about the metabolic state and the existence of various microorganisms (Konig *et al.*, 1994) and in the case of dental caries, porphyrin detection may be a useful indicator of lesion activity, pathogenicity of plaque or the failure of a restoration.

Aims

The aim was to investigate the fluorescent properties of multispecies biofilm developed in the presence of sucrose, and the effect of fluoride exposed continuously, twice or thrice daily and at different concentrations. In addition, the study aimed to investigate the relation between biofilm fluorescence and its cariogenicity, as well as to explore the fluorescent properties of specific bacterial constituents of the biofilm community.

7.2 EXPERIMENTAL PROCEDURE

The biofilm generated over the course of the work of this thesis formed the basis of this study. Immediately after samples were extracted from their respective CDFF experiments, biofilm was photographed intact as described in Section 2.7.4 prior to enumeration of its microbial constituents (Section 2.7.1). Biofilm images were analysed according to the protocol in Section 2.7.4.1 and inter-operator repeatability was investigated.

Preliminary studies used the Inspektor Pro device to image biofilm from a pilot dual CDFF model wherein biofilm was developed (see Section 2.6) on hydroxyapatite and either pulsed with no additional sucrose or 10% sucrose thrice daily. The effect of sucrose on biofilm fluorescence was investigated using the QLF-*in vitro* to image biofilm from the preliminary dual model studies of Chapter 4 (Section 4.3.3). Biofilm was produced on hydroxyapatite and exposed thrice daily to 10% sucrose as described in Section 4.2. The QLF-*in vitro* was also used to investigate the relation between biofilm fluorescence and its cariogenicity by imaging biofilm from the caries model developed in Chapter 3. The correlation of microbial and enamel demineralisation data from these experiments with red, green and red/green fluorescence (R/G) ratios was examined. The influence of continuous fluoride delivery, pulsing twice or thrice daily and fluoride concentration were investigated using the QLF-D by imaging biofilm from the studies presented in Chapter 5 and 6 respectively.

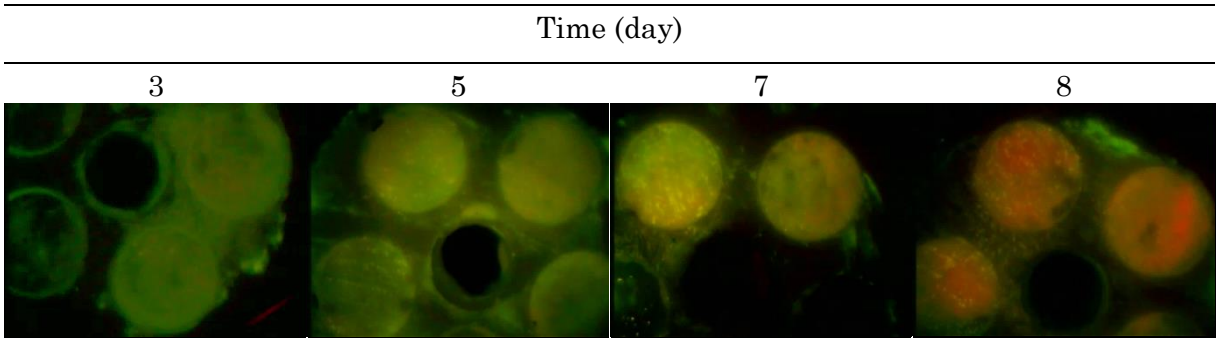
To investigate the fluorescent properties of specific microbial members of the multispecies biofilm, biofilm bacteria were suspended in PBS, serially diluted and cultured on blood agar plates (see Section 2.7.1). Plates were illuminated with QLF light to capture image cubes and fluorescence profiles with multispectral-imaging (CRi Nuance FX Multispectral Imaging Suite, Cambridge Research &

Instrumentation Inc., Woburn, USA). The appearance of specific colonies was noted and categorised as those displaying predominantly red fluorescence, green fluorescence and those that appeared orange. A number of colonies displaying each type of fluorescence were individually sub-cultured by spread plating on blood agar plates and incubated for 24 hours. To determine that pure cultures were isolated, colonies from each spread plate were again sub-cultured onto blood agar plates by streak plating and incubated for 24 hours. Bacterial isolates were frozen with cryopreservative (Microbank™, Prolab Diagnostics, Bromborough, UK) and stored at -20 °C for no more than two months. The identity of these bacterial stocks was investigated using MALDI-TOF and gram stain procedures (see Sections 2.7.1.1 & 2.7.1.2).

7.3 RESULTS

The emergence of red fluorescence as multispecies biofilm matured was first observed in preliminary work visualising biofilm with the Inspektor™ Pro clinical system (Figure 7.1). A combination of green and red fluorescence is noticeable at all times with proportions of red fluorescence predominating by the eighth day in 10% (292 mM) sucrose exposed biofilm. In non-sucrose exposed biofilm, fluorescence remained predominantly green by the eighth day. This equipment was primarily designed to enable the QLF technology to be used clinically. Due to calibration for use in small proximity to surfaces in patients' mouths, focused images could only be captured close to the surface of each CDFF sample pan, meaning the entire pan could not be photographed at one time. The complete illumination of sufficient areas of biofilm limited the objective quantification of biofilm within sample pans from the CDFF model.

Figure 7.1 Longitudinal QLF of multispecies oral biofilm on hydroxyapatite. Images captured using the Inspektor™ Pro system. Preliminary work using the early QLF device primarily used clinically.



7.3.1 The effect of sucrose on multispecies biofilm fluorescence

The QLF-*in vitro* system enabled the capture of high quality images inclusive of the entire area of each sample pan with homogeneous illumination. Significantly higher levels of red fluorescence were observed in biofilm developed in the presence of 10% sucrose over 18 days (Table 7.1 and Figure 7.2). Mean R/G ratios (n = 5) were consecutively higher compared to the non-sucrose pulsed control over the time course. Statistical significance according to the presence or absence of sucrose provided evidence for its positive effect on biofilm red fluorescence. Significance in time as a factor indicated that red fluorescence increased as biofilm matured whilst significance in its interaction with sucrose provided evidence that changes in biofilm fluorescence differ markedly over time in the presence of sucrose (Table 7.1).

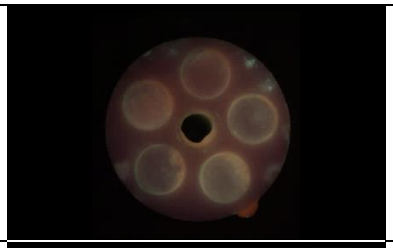
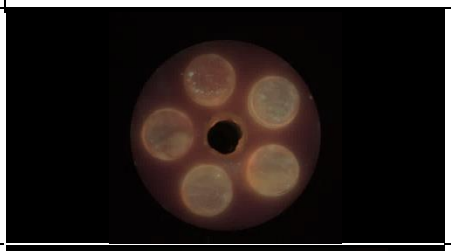
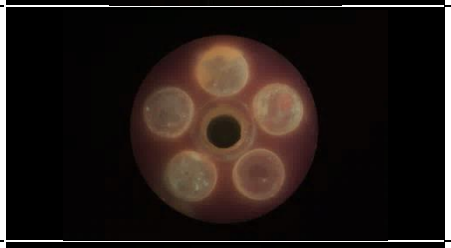
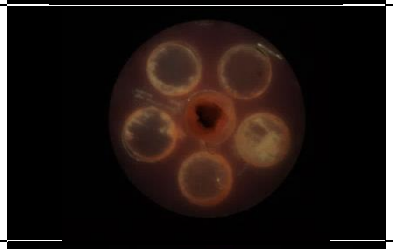
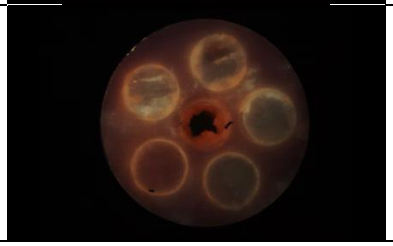
Table 7.1. Results of ANCOVA testing statistical significance of time and sucrose addition on biofilm red fluorescence (R/G) when exposed to 10% sucrose or no sucrose thrice daily

Source of Variation	SSIII	*df*	MS	F	*p*
Sucrose	3.654	2	1.827	286.758	**< 0.001**
Time	0.086	1	0.086	13.462	**0.004**
Sucrose x time	0.043	1	0.043	6.682	**0.027**

R Squared = 0.998 (Adjusted R Squared = 0.997)

Type III sum of squares, SSIII; degrees of freedom, *df*; mean square, MS;
F = (MS between groups)/(MS within groups).
bold font denotes difference is statistically significant.

Figure 7.2 Longitudinal QLF of multispecies oral biofilm on hydroxyapatite expose to no or 10% sucrose. Images captured using the QLF-*in vitro*.

Time (day)	No sucrose	Mean R/G ± SD	10% sucrose	Mean R/G ± SD
4		**1.336** ± 0.132		**1.360** ± 0.116
6		**1.368** ± 0.105		**1.397** ± 0.108
8		**1.469** ± 0.228		**1.421** ± 0.165
11		**1.240** ± 0.159		**1.654** ± 0.149
13		**1.393** ± 0.161		**1.682** ± 0.141
15		**1.432** ± 0.108		**1.557** ± 0.093
18		**1.439** ± 0.229		**1.755** ± 0.084

7.3.2 Biofilm fluorescence in relation to its cariogenicity

Inter-operator repeatability for the method used to analyse the fluorescence characteristics of multispecies oral biofilm in this study was very good; $r = 0.99$ ($p < 0.001$, adjusted $r^2 = 0.97$, $n = 10$; Figure 7.3). The interaction of time with sucrose concentration was statistically significant for changes in green fluorescence ($F(1,8) = 8.042$, $p = 0.022$) and the red/green (R/G) fluorescence ratio ($F(1,8) = 12.711$, $p = 0.007$ but not in red fluorescence ($F(1,8) = 1.604$, $p = 0.241$), when tested using ANCOVA adjusting for time. Thus suggesting that over time fluorescence according to all three characteristics changed differently depending on the concentration of sucrose delivered to the biofilm. Adjusted R^2 values provide evidence that the statistical model accounted for 86.3%, 80.7% and 72.1% regarding green, R/G ratio and red fluorescence respectively. When analysing the dataset as a whole; statistically significant correlations between red, green and R/G ratio fluorescence with cariogenic bacterial constituents of biofilm and enamel demineralisation ranged between 0.480 – 0.698 (Table 7.2) and all can be interpreted as relatively weak. Of statistically significant correlations in viable counts of total bacteria correlation was highest with R/G ratio ($r = -0.679$), followed by green fluorescence ($r = 0.601$) then with red fluorescence ($r = 0.519$). Total *Streptococcus* spp. correlated most well with red fluorescence ($r = 0.698$) followed by green fluorescence ($r = 0.660$) whilst no statistically significant correlations to any fluorescence characteristic was observed in populations of mutans streptococci and *Lactobacillus* spp. Correlations between ΔF, ΔZ and lesion depth were some of the weakest and those for the relationship of these factors individually with R/G ratio were not statistically significant (Table 7.2). However, green fluorescence correlated significantly well with ΔF, ΔZ and lesion depth whilst correlations for red fluorescence with ΔZ were not statistically significant.

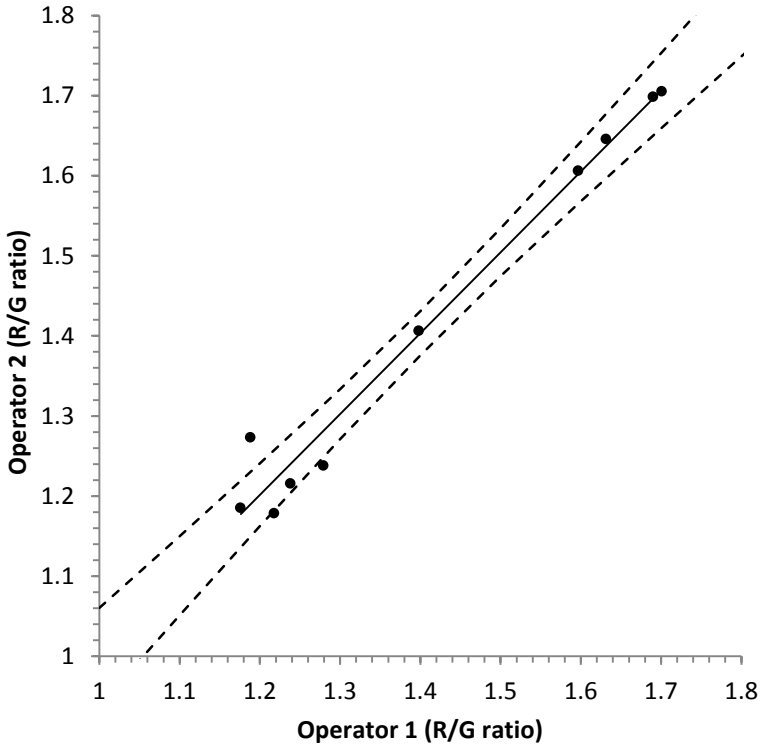


Figure 7.3. Scatter plot of the correlation between two trained operators analysing identical QLF-D images for biofilm red fluorescence in order to determine inter-operator repeatability. Dashed lines represent upper and lower 95% confidence bounds.

Table 7.2. Results of correlation between fluorescence characteristics of biofilm and respective cariogenic features in a biologically relevant caries model pulsed with 20 mM or 100 mM sucrose 8 times daily.

		Biofilm bacterial constituents (log_{10} cfumm^{-2})[a]				Enamel demineralisation characteristics[b]		
		Total bacteria	*Streptococcus* spp.	Mutans streptococci	*Lactobacillus* spp.	abs. ΔF	*ΔZ*	LD (μm)
RF	*r*	**0.519**	**0.698**	-0.102	-0.188	**0.498**	0.452	**0.499**
	p	**0.027**	**0.001**	0.687	0.454	**0.035**	0.060	**0.035**
GF	*r*	**0.601**	**0.660**	-0.147	-0.211	**0.480**	**0.488**	**0.536**
	p	**0.008**	**0.003**	0.562	0.400	**0.044**	**0.040**	**0.022**
R/G	*r*	**-0.679**	-0.408	0.376	0.228	-0.188	-0.342	-0.382
	p	**0.002**	0.093	0.124	0.364	0.456	0.164	0.117

Red fluorescence (RF); Green fluorescence (GF); ratio of red to green pixels (R/G) in images of biofilm imaged using QLF-D. *r* is Pearson's product moment coefficient (n = 18); *p* determines significance of this relationship; emphasised in **bold**

When including data only derived from the 100 mM sucrose pulsed caries model (repeat study) in analysis, the correlations for both mutans streptococci and ΔF with green fluorescence were relatively strong and statistically significant ($r = 0.858$, $p < 0.05$ and $r = 0.860$, $p < 0.05$ respectively); whilst a relatively strong, statistically significant, correlation was observed between red fluorescence and total bacteria. Subsequent linear regression analysis elucidated that the correlation statistic fits these interactions well (adjusted $r^2 = 0.670$, 0.674 and 0.718 respectively); therefore that a low percentage of each can be attributed to variation or unexplainable factors within the model system. In comparison, much lower adjusted r^2 values were found when data from all concentrations were fitted within a linear regression model and these ranged from 0.183 to 0.456.

7.3.3 The effect of fluoride on sucrose fed multispecies biofilm fluorescence

In general, results indicated that proportions of red fluorescence were significantly lower in sucrose pulsed biofilm exposed to additional fluoride than in biofilm exposed to sucrose alone. Table 7.3 summarises the effects of sucrose alone and of additional fluoride on biofilm fluorescence. On the whole QLF-D was able to distinguish a dose-responsive relationship dependent on fluoride concentration and on frequency of exposure. Statistical testing provided evidence that fluorescence significantly differed in response to all fluoride treatments (Table 7.4 – 7.11; $p < 0.001$ in "fluoride" factor) and R^2 values > 0.99 established the suitability of the statistical model selected.

Table 7.3. Mean (± SD) R/G ratios for biofilm exposed to sucrose alone or in combination with fluoride.

Time (day)	**Mean R/G ratio** ± SD (n = 5)						
	100 mM sucrose alone	+ 0.05 ppm F^- continuous	+ 1,450 ppm F^- continuous	+ 228 ppm F^- twice daily	+ 228 ppm F^- thrice daily	+ 1,450 ppm F^- twice daily	+ 1,450 ppm F^- thrice daily
4	**1.224** ± 0.040	**0.943** ± 0.074	**1.099** ± 0.090	**0.957** ± 0.121	**1.054** ± 0.226	**0.948** ± 0.134	**0.902** ± 0.127
6	**1.254** ± 0.101	**1.213** ± 0.011	**1.206** ± 234	**1.059** ± 0.264	**1.015** ± 0.234	**0.910** ± 0.168	**0.840** ± 0.136
8	**1.264** ± 0.064	**1.159** ± 0.030	**1.153** ± 0.286	**1.075** ± 0.234	**1.022** ± 0.212	**0.908** ± 0.108	**0.839** ± 0.102
10	**1.384** ± 0.066	**1.067** ± 0.040	**1.528** ± 0.404	**1.176** ± 0.200	**0.974** ± 0.170	**0.946** ± 0.125	**0.929** ± 0.087
12	**1.604** ± 0.123	**1.227** ± 0.022	**1.498** ± 0.820	**1.090** ± 0.253	**0.987** ± 0.170	**0.970** ± 0.043	**1.008** ± 0.074
14	**1.404** ± 0.122	**1.081** ± 0.070	**1.554** ± 0.401	**1.115** ± 0.208	**0.995** ± 0.180	**1.054** ± 0.101	**1.001** ± 0.158
Av.	**1.356** ± 0.142	**1.115** ± 0.107	**1.308** ± 0.216	**1.079** ± 0.072	**1.008** ± 0.029	**0.956** ± 0.054	**0.920** ± 0.074

Red fluorescence was significantly reduced when a third application of fluoride was administered compared to twice daily in both fluoride concentrations (Table 7.3, 7.4 & 7.5). 1,450 ppm fluoride delivered thrice daily appeared to exert the most reductive effect on biofilm red fluorescence. Statistical significance according to the interaction of time and fluoride frequency, in differences between fluorescence in biofilm exposed to 228 ppm fluoride twice or thrice daily (Table 7.4) suggests that fluorescence changed in markedly different manners in each condition. In biofilm pulsed with 228 ppm fluoride thrice daily red fluorescence was successively lower over time than in twice daily exposed biofilm (Table 7.3). Significance in time and not the interaction with fluoride for 1,450 ppm fluoride exposed biofilm either twice or thrice daily (Table 7.5) indicates that fluorescence decreased over time in both but in similar ways regardless of the frequency of exposure. When comparing red fluorescence in biofilm

pulsed thrice daily with 228 ppm or 1,450 ppm fluoride the significant interaction between time and fluoride concentration (Table 7.7) indicates that these concentrations delivered three times in each day differentially affect the biofilm and over time changes were markedly different. When exposed twice daily with either concentration, significance in the factor of time (Table 7.6) indicates that fluorescence significantly decreased over time in both but was not differentially affected when these concentrations were delivered twice daily. Significance in the interaction between fluoride and time when comparing biofilm exposed to 228 ppm fluoride thrice daily or 1,450 ppm fluoride twice daily (Table 7.9) provides further evidence that increasing fluoride concentration significantly reduces biofilm red fluorescence even when lower concentrations of fluoride are delivered three times daily. However, no significance in this interaction and significance only in time when comparing red fluorescence in biofilm pulsed thrice daily with 1,450 ppm fluoride or with 228 ppm fluoride twice daily (Table 7.8), indicates that though fluorescence significantly reduced with time, raising the concentration and frequency of delivery may not indefinitely reduce red fluorescence. The results indicate that biofilm red fluorescence differed significantly in the presence or absence of 0.05 ppm fluoride (Table 7.10). However, no significance over time alone and its interaction with fluoride suggests that low level fluoride did not elicit changes in fluorescence over time and nor in a differential manner to 100 mM sucrose alone. Continuous exposure to 1,450 ppm fluoride appeared to significantly influence biofilm red fluoresce compared to 100 mM sucrose alone (Table 7.11), yet it appears red fluorescence was higher in fluoride exposed biofilm than in sucrose alone by the end of the time course (Table 7.3). The interaction of time with fluoride was not significant (Table 7.11) providing evidence for the similarities in changes over time under either condition. Furthermore, red fluorescence was, on average, higher here than when fluoride was delivered intermittently either twice or thrice daily (Table 7.3).

Table 7.4. Results of ANCOVA testing statistical significance of time and fluoride addition on biofilm red fluorescence (R/G) when exposed to caries challenge and **228 ppm fluoride twice or thrice daily.**

Source	SSIII	*df*	MS	F	*p*
Fluoride	1.530	2	0.765	481.407	**< 0.001**
Time	0.003	1	0.003	1.795	0.217
Fluoride x time	0.016	1	0.016	9.925	**0.014**

R Squared = 0.999 (Adjusted R Squared = 0.999)

Table 7.5. Results of ANCOVA testing statistical significance of time and fluoride addition on biofilm red fluorescence (R/G) when exposed to caries challenge and **1,450 ppm fluoride twice or thrice daily.**

Source of Variation	SSIII	*df*	MS	F	*p*
Fluoride	1.018	2	0.509	237.810	**< 0.001**
Time	0.024	1	0.024	11.263	**0.010**
Fluoride x time	0.001	1	0.001	0.388	0.551

R Squared = 0.998 (Adjusted R Squared = 0.998)

Table 7.6. Results of ANCOVA testing statistical significance of time and fluoride addition on biofilm red fluorescence (R/G) when exposed to caries challenge and **228 ppm or 1,450 ppm fluoride twice daily.**

Source of Variation	SSIII	*df*	MS	F	*p*
Fluoride	1.235	2	0.618	281.059	**<0.001**
Time	0.023	1	0.023	10.612	**0.012**
Fluoride x time	0.001	1	0.001	0.314	0.590

R Squared = 0.999 (Adjusted R Squared = 0.998)

Table 7.7. Results of ANCOVA testing statistical significance of time and fluoride addition on biofilm red fluorescence (R/G) when exposed to caries challenge and **1,450 ppm or 228 ppm fluoride thrice daily.**

Source of Variation	SSIII	*df*	MS	F	*p*
Fluoride	1.313	2	0.656	428.533	**< 0.001**
Time	0.003	1	0.003	2.044	0.191
Fluoride x time	0.016	1	0.015	10.720	**0.011**

R Squared = 0.999 (Adjusted R Squared = 0.998)

Type III sum of squares, SSIII; degrees of freedom, *df*; mean square, MS;
F = (MS between groups)/(MS within groups).
bold font denotes difference is statistically significant.

Table 7.8. Results of ANCOVA testing statistical significance of time and fluoride addition on biofilm red fluorescence (R/G) when exposed to caries challenge and **228 ppm fluoride twice daily or 1,450 ppm fluoride thrice daily.**

Source	SSIII	*df*	MS	F	*p*
Fluoride	1.136	2	0.568	207.356	**< 0.001**
Time	0.033	1	0.033	12.027	**0.008**
Fluoride x time	6.429×10^{-6}	1	6.429×10^{-6}	0.002	0.963

R Squared = 0.998 (Adjusted R Squared = 0.997)

Table 7.9. Results of ANCOVA testing statistical significance of time and fluoride addition on biofilm red fluorescence (R/G) when exposed to caries challenge and **228 ppm fluoride thrice daily or 1,450 ppm fluoride twice daily.**

Source of Variation	SSIII	*df*	MS	F	*p*
Fluoride	1.412	2	0.706	713.547	**< 0.001**
Time	0.001	1	0.001	0.744	0.413
Fluoride x time	0.010	1	0.010	9.970	**0.013**

R Squared = 0.998 (Adjusted R Squared = 0.998)

Table 7.10. Results of ANCOVA testing statistical significance of time and fluoride addition on biofilm red fluorescence (R/G) when exposed to **100 mM sucrose intermittently alone or with additional 0.05 ppm fluoride continuously.**

Source of Variation	SSIII	*df*	MS	F	*p*
Fluoride	1.702	2	0.052	74.871	**< 0.001**
Time	0.052	1	0.015	4.614	0.064
Fluoride x time	0.015	1	0.011	1.285	0.290

R Squared = 0.995 (Adjusted R Squared = 0.993)

Table 7.11. Results of ANCOVA testing statistical significance of time and fluoride addition on biofilm red fluorescence (R/G) when exposed to **100 mM sucrose intermittently alone or with additional 1,450 ppm fluoride continuously.**

Source of Variation	SSIII	*df*	MS	F	*p*
Fluoride	1.464	2	0.732	64.780	**< 0.001**
Time	0.203	1	0.203	17.942	**0.004**
Fluoride x time	0.013	1	0.013	1.177	0.314

R Squared = 0.996 (Adjusted R Squared = 0.994)

Type III sum of squares, SSIII; degrees of freedom, *df*; mean square, MS;
F = (MS between groups)/(MS within groups).
bold font denotes difference is statistically significant.

7.3.4 The fluorescent properties of specific biofilm bacteria

Microorganisms from the multispecies biofilm generally fluoresced red, green or a combination of the two when cultured on agar containing blood and illuminated with QLF light (Figure 7.4). In colonies predominantly red in appearance, multi-spectral imaging elucidated the presence of a dominant peak at 635 nm accompanied with a shoulder and peak at 720 nm (Figure 7.4; pink arrow). A broad moderate peak in emission in the green light wavelength with emission extending into the yellow wavelength (520 – 570 nm) was also observed from these colonies. A peak in this range was the only one in green fluorescent colonies (Figure 7.4; cyan arrow) but its intensity was higher and encompassed a broader range of wavelengths (490 – 610 nm). No peak at 635 nm explains the predominance of green in this isolate. Fluorescence at 635 nm with a peak at 720 nm is attributable to the production of protoporphyrin IX (Konig, 1993; Seo, 2009). Subsequent MALDI-TOF MS identified red fluorescent isolates as *Lactobacillus rhamnosus*, *Veillonella parvula* and a viridans α-haemolytic streptococcus whilst green fluorescent isolates were identified as *S. mutans* and *L. rhamnosus* (Table 7.12). Figure 7.5 summarises Gram stains. Applying these spectra onto the agar plate image (Figure 7.4) assigned false colours (pink, predominantly red fluorescent; yellow, moderately orange; cyan, green fluorescent colonies) to areas exhibiting the same wavelength of light (Figure 7.6a). It emphasised that bacteria of this model emitted a combination of fluorescence attributes. It also elucidated that red fluorescence in visibly red colonies was centralised to the core of the colony with moderately orange characteristics forming a ring on the outer portions. Orange fluorescence can be interpreted as the presence of some green fluorescent metabolic products along with those red fluorescent by nature in these regions, and its combined effects on the emitted wavelength. This form of image analysis could be applied onto images of other biological tissues to characterise differential fluorescence more vividly. It elucidated the highly heterogeneous nature of fluorescence in multispecies biofilm (Figure 7.6b) and the retention, and thereby

the penetration, of fluorescent metabolic products of bacterial metabolism into enamel surfaces (Figure 7.6c). Here, intense red fluorescence with protoporphyrin IX peak was assigned the false colour red; orange spectra with moderate protoporphyrin IX peak assigned white; green biofilm spectra assigned cyan and green enamel spectra assigned deep blue.

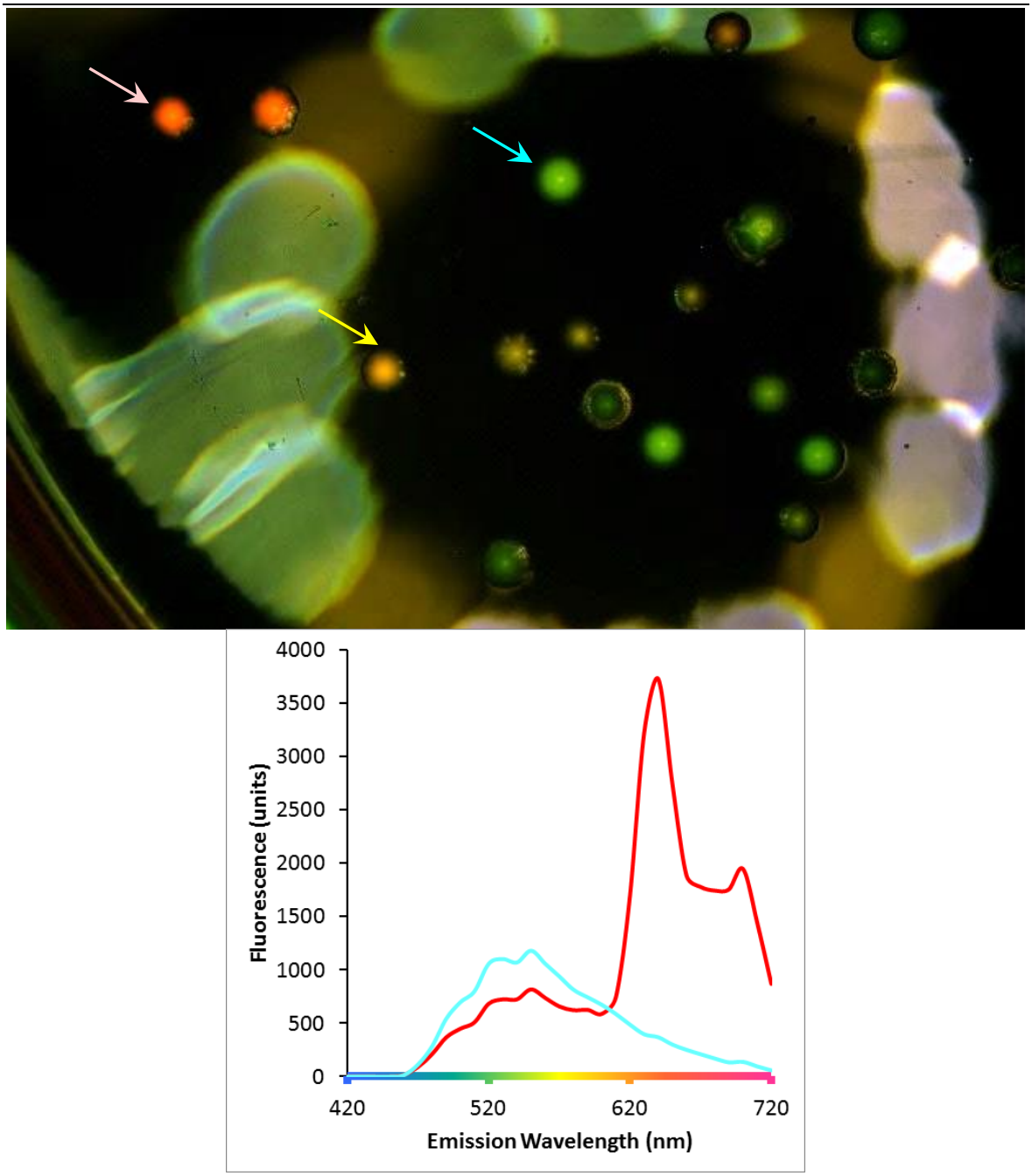


Figure 7.4. Bacterial colonies on blood agar plates imaged using MSI and illuminated with 405 nm QLF light.

Table 7.12. MALDI-TOF identification of red and green fluorescent bacteria from multispecies biofilm with observations of colony morphology and gram stains.

MALDI-TOF Identification	Predominant Fluorescence	Colony morphology*	Gram stain observations
S. mutans	Green	Small, dry	positive coccus
L. rhamnosus	Green	Large, wet	positive bacillus
L. rhamnosus	Red	Large, circular	positive bacillus
Viridans a haemolytic strep.	Red	Large, flat	positive coccus
Veillonella parvula	Red	Small, heaped	negative coccus

*on blood base no. 2 agar supplemented with 5% defibrinated horse blood

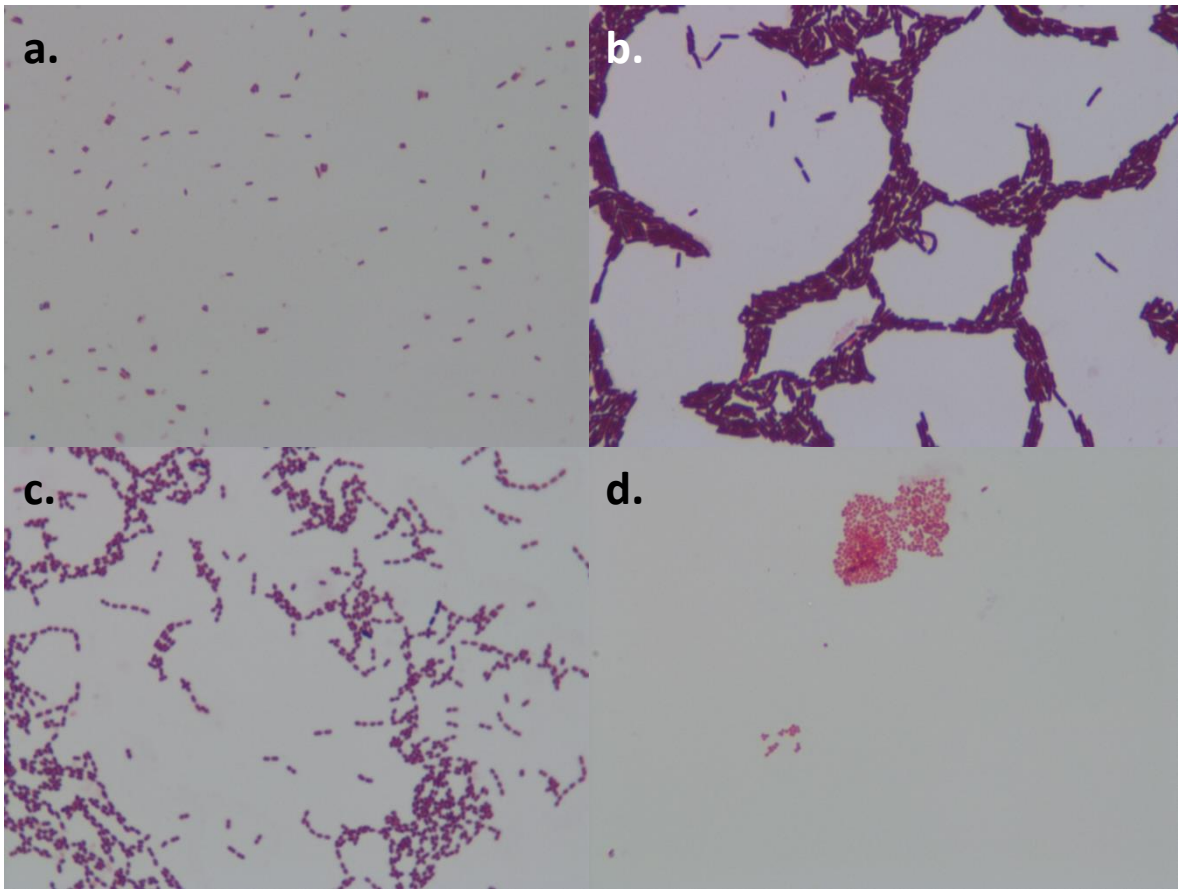


Figure 7.5. White light microscopic images of Gram-stained bacteria (100x magnification) identified with MALDI-TOF MS (see Table 7.12). (a) *S. mutans*; (b) *L. rhamnosus*; (c) *viridans streptococci*; (d) *Veillonella parvula*.

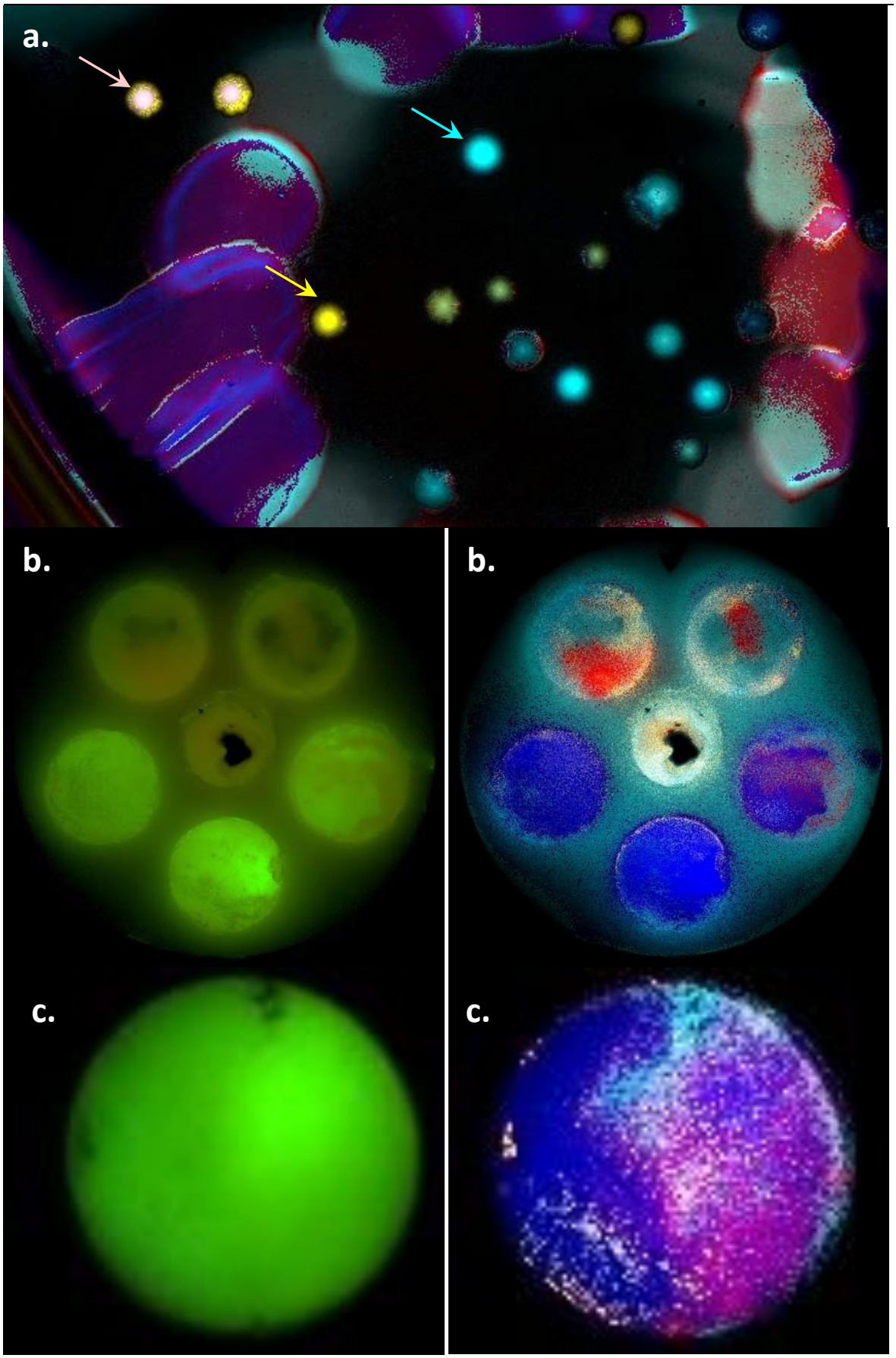


Figure 7.6. Differentiation of defined spectral fluorescence in (a) bacterial colonies (b) CDFF biofilm sample pan and (c) enamel post biofilm exposure imaged by MSI and illuminated with 405 nm QLF light.

7.4. DISCUSSION

The work of this study provides evidence for the use of QLF to elucidate the metabolic activity of multispecies biofilm. Early work identified the presence of green and red fluorescence in multispecies biofilm and that the red fluorescent properties increased as biofilm matured. However, the Inspektor pro clinical system has limitations for use *in vitro*. Building on early observations indicating that sucrose may favourably stimulate red fluorescence leading to higher levels as biofilm matured was investigated using the further developed QLF products; QLF-*in vitro* and QLF-Biluminator. Red fluorescence (R/G ratio) was successively higher over time in biofilm exposed to 10% sucrose thrice daily compared to non-sucrose pulsed biofilm. Red fluorescence as an indication of biofilm maturation agrees with the work of others wherein biofilm was developed over 10 days with continuous 0.5% sucrose (Kim *et al.*, 2014) and over 14 days in a cross-over *in situ* study (Bittar *et al.*, 2014). The latter also observed significantly higher levels of red fluorescence in the sucrose group. The repeatability of the method employed in the current study for characterising the fluorescence of multispecies oral biofilm was confirmed. The correlation testing for paired inter-operator measurements was strong ($r = 0.99$) and was supported statistically ($p < 0.001$, adjusted $r^2 = 0.97$, $n = 10$).

Recent research supports the theory that red fluorescence observed in dental plaque is likely related to the intrinsic metabolic products of the oral biofilm on a whole, and not inherently to the presence of the specific bacteria (Volgenant *et al.*, 2013). In concordance, the current study revealed that *Lactobacillus rhamnosus* were able to exhibit both predominantly green and predominantly red fluorescence; likely a consequence of their differential metabolic pathways in response to the various micro-environments in plaque or changing conditions of different regions of the biofilm. It is possibly also a consequence of whether either bacterium was in synergism with other members of the microbial community forming food chains to facilitate its metabolism

of available nutrients and for example, its production of protoporphyrin IX at the very least. It has so far been demonstrated in one study that *Parvimonas micra* fluoresces red only in the immediate presence of *Porphyromonas gingivalis* (van der Veen *et al.*, 2006). This and the current study indicate that further investigations of how the interactions of specific bacteria may influence red fluorescence would be worthwhile.

While *S. mutans* was determined as predominantly green fluorescent in this study, bacteria identified by MALDI-TOF MS as viridans α-haemolytic streptococci were principally red fluorescent. The use of MALDI-TOF MS for easy routine identification of bacteria and yeasts has been demonstrated, however, the correct identification of viridans group streptococci has proved problematic (van Veen *et al.*, 2010; Steensels *et al.*, 2011) whilst 100% consistency was also observed in species differentiation in this group (Friedrichs *et al.*, 2007). Due, in part, to recombination between closely related species, bacteria of this group are notoriously resistant to classification. Nonetheless, the isolate of the current study has the potential to be a recombinated *S. mutans* analogue or one of the other members of the mutans streptococci group such as; *S. mitis*, *S. oralis*, *S. sanguinis*, and *S. sobrinus*. Subsequent resistance to optochin would rule out its indentity as *S. pneumoniae* in the very least.

If red fluorescence is viewed as an indicator of bacterial metabolism the results of the current study provided evidence for the antibacterial effects of fluoride. Accordingly, the study also contributes to the notion that red fluorescence is related to bacterial metabolism. Fluoride is known to exert a number of direct and indirect effects on bacteria to inhibit central cellular processes (Section 1.5.3). In the context of its anti-caries activity its importance as an antimicrobial seems most related to its demonstrable influence on bacterial acid production (Section 1.5.3.2). In this regard, visualising the red fluorescent properties of the biofilm has contributed to our

understanding of its differential effects on bacteria irrespective of its demonstrated effects on enamel demineralisation discussed in the earlier portions of this thesis.

In general, all treatments of fluoride reduced biofilm red fluorescence in comparison to fluorescence in biofilm exposed to sucrose alone, with an evident dose-response as fluoride concentration and frequency of application increased. A third daily application of fluoride consistently reduced biofilm red fluorescence compared to when fluoride of either concentration was supplied twice daily. 1,450 ppm fluoride delivered thrice daily appeared to exert the most reductive effect on biofilm red fluorescence. Since only time and fluoride as separate factors were statistically significant, it appears red fluorescence was decreased in both frequencies but that the change over time occurred similarly. This could suggest that the influence of 1,450 ppm fluoride on bacterial metabolism has an upper limit of efficacy. This limit is further suggested by results in biofilm exposed to 1,450 ppm fluoride continuously whereby red fluorescence was higher than in sucrose exposed biofilm by the end of the time course. Nonetheless, it appears continuous 1,450 ppm fluoride inhibited red fluorescence earlier in the experiment, indicating that some members of the multispecies biofilm may be capable of adapting to its uninterrupted presence and continue to produce these fluorescent metabolic products. Changes in fluorescence over time were similar to those exhibited in biofilm exposed to sucrose alone. Furthermore, red fluorescence was, on average, higher here than when fluoride was delivered intermittently either twice or thrice daily, providing insight into the potential efficacy of this approach in treatment. Differences in red fluorescence in biofilm exposed twice or thrice daily to 228 ppm fluoride differed in marked ways over time, confirming again the potential benefit of the third application of 1,450 ppm (once slurried) or 228 ppm dentifrice. When pulsed twice daily with either concentration of fluoride, it appears that a difference could not be distinguished based on the interaction of concentration and time. However, fluorescence decreased over

time in both and fluoride exerted reductive effects on red fluorescence. It appears that pulsing thrice daily with either concentration of fluoride exerted markedly differential effects on the emission of red fluorescence which changed differently over time. Whereas, two times daily exposure to 1,450 ppm fluoride significantly reduced biofilm red fluorescence even when lower concentrations of fluoride are delivered thrice daily. However, raising the concentration and frequency of delivery of fluoride may not indefinitely reduce red fluorescence since it changed in similar manners over time when biofilm was exposed three times daily to 1,450 ppm fluoride or twice daily to 228 ppm fluoride. Nonetheless, the reductive effect was significantly greater at the higher concentration but these results inherently reaffirm the earlier interpretation that the efficacy of fluoride may have an upper limit in this context. Finally, low level fluoride appeared to exert a subtle effect on biofilm fluorescence however it did not elicit significant changes in fluorescence over time and in a differential manner to 100 mM sucrose alone. Importantly; this coincides with the lack of significant differences in the demineralisation of enamel in either condition as reported in Chapter 5 (Section 5.3.1 and 5.4.1).

7.4.1 Biofilm fluorescence in relation to its cariogenicity

Much research has focused on the potential application of QLF light in determining caries risk by quantifying biofilm red fluorescence. So far, it has been found that the presence or absence of red fluorescence is a good indicator of general oral health and the specific intelligent design of the QLF™-D products aids in patient understanding (Waller *et al.*, 2003). Others have confirmed that there is a significant association between the red fluorescence (expressed as R/G ratio) and the cariogenic properties of microcosm oral biofilm visualised *in vitro* using QLF™D (Lee *et al.*, 2013) as well as that this technology shows promise in monitoring the maturation of microcosm oral biofilm (Bittar *et al.*, 2014; Kim *et al.*, 2014). Though red fluorescence significantly increased over time *in situ*, both sucrose and non-sucrose exposed samples exhibited

this trend and mineral loss over time was significant only in the sucrose exposed group (Bittar *et al.*, 2014). This finding indicates that red fluorescent dental plaque is not necessarily associated with its cariogenicity. The present study revealed that red fluorescence (as R/G ratio) is not always well associated with factors of the caries disease process. In cases of highly heterogeneous levels of enamel demineralisation the fluorescent properties of multispecies biofilm are not an appropriate indicator of its cariogenicity, when based solely on the linear correlation of bacterial quantities and determinants of enamel mineralisation with fluorescence intensity. However, stronger correlations and straight line relationships between these factors were obtained in cases with significant caries activity. In this regard, further research is required and the development of more appropriate statistical model approaches would be invaluable to this field of research. Nonetheless, there is mounting evidence that QLF is highly suitable for the purpose of quantifying dental plaque and improving oral hygiene by educating, hence improving, patient compliance.

ANCOVA models examined the effect of sucrose concentration and time on red and green fluorescence and the red/green (R/G) ratio of the two. Statistically significant interactions between the effects of sucrose and time on green fluorescence and R/G ratio indicate that these fluorescence characteristics changed differently over time depending on the concentration of sucrose delivered to the biofilm. This may be an indication of the differential bacterial metabolism in response to sucrose availability theorised earlier in the discussions of this thesis (Section 3.4.1). In 100 mM sucrose exposed samples the correlations of green fluorescence with mutans streptococci, as well as with QLF fluorescence loss as an indicator of caries progression, were strong (r = 0.9, p = 0.03, adj. r^2 = 0.7 and r = 0.9, p = 0.03, adj. r^2 = 0.7 respectively). The former correlation may be attributed to earlier findings that *Streptococcus mutans* predominantly exhibits green fluorescence (Konig *et al.*, 1993; Lennon *et al.*, 2006). This bacterial group is notably capable of synthesising EPS in the presence of sucrose

(Koo *et al.*, 2003; Koo *et al.*, 2005). The sucrose dose-responsive growth trends of mutans streptococci observed in the present study (see Chapter 3), in line with the relationship between EPS production and sucrose concentration demonstrated previously (Cury *et al.*, 1997; Cury *et al.*, 2000; Ribeiro *et al.*, 2005; Aires *et al.*, 2006) provide a rational explanatory for the strong positive correlation between green fluorescence and ΔF. Moreover, under these conditions proportions of mutans streptococci were the only bacterial group within the multispecies biofilm that correlated strongly with ΔF significantly well ($r = 0.9$, $p = 0.03$, adjusted $r^2 = 0.7$). Overall this series of statistical analyses reveals that as proportions of mutans streptococci increased, green fluorescence within biofilm increased and, most likely as a consequence of bacterial metabolism, enamel demineralisation increased as indicated by enamel fluorescence loss assessed by QLF.

Furthermore, the relationship between red fluorescence alone and total bacteria was strong and statistically significant ($r = 0.9$, $p = 0.02$, adjusted $r^2 = 0.7$) in samples pulsed with 100 mM sucrose. Red fluorescence did not correlate statistically significantly with ΔF however, whilst R/G ratio did ($r = 0.9$, $p = 0.01$, adjusted $r^2 = 0.8$). The statistical significance of the latter could be attributed to the co-operative influence of the green and red fluorescent bacteria/bacterial products that impact the enamel demineralisation. Although no statistically significant correlation between total bacteria and R/G has been reported (Lee *et al.*, 2013), it would be short sighted to insinuate that the findings of the present work disagree. Lee *et al* (2013) also showed that R/G significantly correlated with aciduric bacteria ($r = 0.83$, $p < 0.0001$). It has already been established in the present study that biofilm undergoes rapid acidification in response to the presence of 100 mM sucrose (see Section 3.3.5 and 3.4.1) and that members of the microbial community are capable of persisting in this environment. It is even likely that this environment selects for the proliferation of aciduric species. Therefore, it is plausible to suggest that the current study confirms

the findings of this previous work as well as that of Kim *et al* (2014) who reported statistically significant correlations between R/G and total bacteria ($r = 0.74$, $p = 0.001$) and aciduric bacteria ($r = 0.85$, $p = 0.001$) exposed to 0.5% (14.6 mM) sucrose.

When pooling data from all sucrose pulsed experiments, unlike previous work (Lee *et al.*, 2013; Kim *et al.*, 2014), correlations of R/G ratio with enamel demineralisation (ΔF, ΔZ and lesion depth) were not statistically significant and among the weakest of all relationships examined. ΔF and lesion depth, exhibited poor correlations with red ($r = 0.5$ in both) and green ($r = 0.5$ in both) fluorescence and were statistically significant. Correlations of mineral loss (ΔZ) were statistically significant only with green fluorescence but again reflected little relationship between these factors ($r = 0.5$). Furthermore, Kim *et al.* (2014) cite the statistically significant ($p = 0.001$) correlation of R/G with enamel demineralisation according to surface microhardness and lesion depth as $r = 0.65$ and 0.82 respectively which are, nonetheless, weak. The weak correlations (defined statistically as $r < 0.9$) of the current study can be partially attributed to the inherent variability of biological model systems, the caries they produce and traditional microbiological culture techniques which may weaken this statistical test. Consequently, calling into question its appropriateness in studies that aim to prove intrinsic associations. r^2 values derived from linear regression analyses provide an effective guide for interpreting the fitness of statistical models, regardless of statistical significance, and is unfortunately underused or often not cited in studies of this type. The low r^2 values, when including all available paired data in the current study, can be further attributed to the non-statistically significant correlations between all fluorescent properties and the cariogenic properties observed in the 20 mM sucrose data subset. It is also worth noting the similar influence of data from these samples in previous sections of this chapter (see 3.3.6 and 3.4.2) established as likely due to the lack of caries lesions and problems with TMR. With this subset included, r^2 values indicated that, despite statistical significance for the correlation of

a number of variables, the correlation statistics account for between 18.3% and 45.6% of the data. When correlation and regression analysis within data from the 100 mM sucrose exposed samples alone (wherein, significant enamel demineralisation occurred as early as day 4 and continued throughout; see Section 3.3.7) were employed, correlations were both the strongest obtained and improved r^2 values reflected that the correlation statistics fit the interactions markedly better. In the latter regard, a lower percentage (> 33% compared to > 81.7% with 20 mM) of each can be attributed to variation or factors not investigated within the model system.

The correlation between total bacteria and R/G ratio when using data from all sucrose pulsed experiments reported in the present study (r = -0.68) is similar in strength to work by others (r = 0.74, Kim *et al*, 2014), however it indicates an inverse relationship between these variables, suggesting that as total bacteria increased, R/G on a whole decreased. Nonetheless, the statistically significant relationship between total bacteria and red (r = 0.52) and green (r = 0.60) fluorescence separately were each positive indicating a straight line relationship between total bacteria weakly associated with each type of fluorescence. It would appear that as total bacterial counts increased, it is likely that the intensities of both red and green fluorescence increased. The simultaneous increase of both types of fluorescence can contribute to the R/G ratio so that when green fluorescence is particularly high the presence of red fluorescence may be made diminutive when expressed within the R/G ratio, conventionally cited by others in this field. It would appear that the methodology used in the work of Lee *et al* (2013) and Kim *et al* (2014) gave rise to distinctly high levels of red fluorescence from the biofilms developed; images of biofilm included in these publications confirm this. This could be attributed to the inclusion of a precursor to vitamin K, menadione, in the artificial saliva medium, since it has been shown that a number of oral bacteria can be induced to fluoresce red in the presence of vitamin K to form metal-free porphyrin (Dolowy *et al.*, 1995; Volgenant *et al.*, 2013).

This includes the aciduric bacteria *S. mutans*. The inverse relationship between total bacteria and R/G ratio observed in the present study could also be attributed to the thickness of biofilm and the impact of fluorescent quenching. The previous biofilm studies could not report biofilm thickness likely due to the multi-well biofilm model approach employed (Lee *et al.*, 2013; Kim *et al.*, 2014). This may impact on how representative these results are of the natural situation. The quenching of fluorescence refers to a variety of processes capable of decreasing the fluorescence intensity of a substance; including processes of photochemical decomposition, stimulated light scattering, energy transfer, complex-formation, influences of solvents in the microenvironment, excited state reactions, induced transitions to the ground state and collision based quenching (Selwyn and Steinfeld, 1969). Acrylamide, iodide and chloride ions as well as molecular oxygen are commonly regarded chemical quenchers (Lakowicz and Weber, 1973; O'Reilly, 1975; Phillips *et al.*, 1986; Sacksteder *et al.*, 1990; Gutow, 2005). Excited state absorption in large molecules leads to a decrease in the primary quantum yield of a photochemical or photophysical process and quantum yield decreases with increasing light intensity (Speiser *et al.*, 1973). In their excited state molecules re-absorb a photon causing a photoquenching effect and, in general, this photon is converted into heat via a non-radiation process. The consequence of these processes is that photons affected do not contribute to the yield (Speiser *et al.*, 1973). The photoquenching phenomenon can be attributed to the occurrence of stimulated transitions from the excited level to high vibrational sub-levels of the found state (Selwyn and Steinfeld, 1969). It has been extensively shown that photoquenching influences practically all luminescence and spectroscopic characteristics of a substance such as intensity, lifetime, quantum yield, spectrum, and anisotropy of the radiation (Rehm and Weller, 1970; Lakowicz and Weber, 1973; Speiser *et al.*, 1973). On this basis it is at least likely that the presence of a diverse set of molecules within micro-environments within the biofilm could influence the neighbouring fluorescence and the manifestation of biofilm fluorescence on a whole.

Taking into account the trademark multispecies co-operation observed in dental plaque is an important factor when investigating the fluorescence phenomena within the oral biofilm setting.

7.5 CONCLUSIONS

Overall, the current study provided evidence of biofilm red fluorescence as an indicator of bacterial metabolism and biofilm maturation. Sucrose exposed biofilm emitted successively higher levels of red fluorescence while fluoride inhibited increases in red fluorescence over time. It also revealed that bacteria may differentially exhibit proportions of both green and red fluorescence when in community and complex microbial interactions may exert some effect on fluorescence characteristics. With regards to the theory that biofilm red fluorescence can be related to its cariogenicity QLF of multispecies biofilm revealed strong significant correlations and straight line relationships between enamel demineralisation and the fluorescent properties of biofilm exposed to 100 mM sucrose; wherein caries progression was revealed to be high and onset was fast. However, in cases of highly heterogeneous levels of enamel demineralisation (20 mM sucrose exposed) the fluorescent properties of multispecies biofilm were not an appropriate indicator of its cariogenicity, when based solely on the linear correlation of bacterial quantities and determinants of enamel mineralisation with fluorescence intensity. In this regard, red fluorescence (as R/G ratio) is not always well associated with factors of the caries disease process. Further investigation is required and the development of more appropriate statistical model approaches would be invaluable to this field of research.

Chapter 8 General discussion, conclusions and future work

It has long been appreciated that not all dental plaque is removed from tooth surfaces after brushing, it is therefore important to elucidate the risk or progression of caries at these sites. Dental plaque and enamel as reservoirs for fluoride have been revealed (Duckworth *et al.*, 1989; Vogel *et al.*, 1992a; Vogel *et al.*, 1992b; Zero *et al.*, 1992) and could prove to be important aspects of the anti-cariogenic feature of fluoride by maintaining elevated levels of fluoride at the tooth surface when sugars are consumed. Furthermore, continuous interest in developing novel anti-caries agents has generated a need for biologically representative laboratory models capable of assessing their anti-caries effectiveness. The CDFF has been used extensively in dental research. To date, however, the majority of studies modelled biofilm alone and in this regard, such research contributed to the understanding of only one aspect of the caries process. In terms of *in vitro* research, pH cycling models in particular have greatly contributed to our understanding of the enamel related aspect of caries. Yet non-biological models do not fully represent the mix of organic acids, nor do they account for the challenge faced by therapeutic agents in penetrating biofilm to reach enamel and aid in its remineralisation. Caries is the localised demineralisation of tooth enamel when biofilm bacteria metabolise dietary carbohydrates into acids. It is, therefore, important to employ a holistic approach in its study whereby the cariogenicity of biofilm alongside the progression of demineralisation in enamel is investigated. However, in reality; few models incorporate such an approach.

8.1 Assessing the role of fluoride in caries control

The caries model developed here enabled the study of the anti-caries effects of fluoride in an attempt to assess its efficacy under conditions simulating the modern diet. The current methodology innovatively employed the CDFF model to investigate the caries disease process in response to fluoride delivered continuously; twice and thrice daily; and at different concentrations. The approach is the first in CDFF research to model caries inclusive of a biologically relevant microcosm biofilm in addition to the progression of demineralisation in enamel. The current work focused on the quantification of a few members of the microbial community within the multispecies biofilm using traditional microbiological culture techniques, whilst also quantifying caries characteristics with 'gold standard' (TMR), bourgeoning (QLF), and novel (NCSP) procedures. However, this model could be examined using an array of techniques to obtain information about a number of aspects of the biofilm; information about the surface of the substratum; and to validate other upcoming methods of quantifying changes within the substratum. In this regard, the current study contributes not only to enamel caries research but to biofilm research in general. Within dental research, for example, the model could be further developed for the study of subgingival caries and erosion by changing procedural conditions. With an ageing dentate population, the relationship between diet and root caries requires ever more attention. It could also be expanded to investigate occlusal caries as these sites are known to be more vulnerable to the accumulation of biofilm. Used in conjunction with 3D-cell culture models, the current technology could be incorporated into programmes of research to investigate aspects of the periodontal disease process.

A core finding of the current study indicates that quantities of total or specific members of the microbial community cannot be deemed as direct indicators of caries activity or risk. This is in agreement with prevailing opinions on the way we should view the relation between biofilm microbiology and caries (Marsh, 1994, 2003a;

Beighton, 2005). Throughout the work, the numbers of bacteria commonly associated with caries were not always directly related to the level of demineralisation observed in enamel beneath biofilm. Exposure to differential sucrose concentrations alone, as well as in combination with fluoride via different means, demonstrated that understanding the metabolism of oral biofilm bacteria, and how it may be affected, is vital to our understanding of caries. Promising avenues to shed further light on the biofilm findings of the current work include; directly quantifying EPS production; exploring metagenomic, metatranscriptomic, metabolomic and metaproteomic profiles of differentially treated biofilm; as well as extracting plaque fluid to investigate the identity and proportions of acids and to study its mineral saturation and fluoride content via capillary electrophoresis and micro fluoride probes respectively. Improving on the design of the system may be possible so that factors such as pH and oxygen gradients can be longitudinally monitored *in situ*, or these factors could be investigated using novel nano-probes. The model could be applied to explore the use of nano-probe technology within biologically relevant biofilm. The structure of biofilm has been extensively reported using approaches such as SEM (Nyvad and Fejerskov, 1987, 1989, 1994; Ramage *et al.*, 2010) and could also be investigated along with indications of the metabolism of biofilm constituents using confocal imaging in conjunction with pH measures. The complexity of these biofilms could also be characterised with approaches such as qPCR and PCR-DGGE, the application of which has been previously demonstrated (Pratten *et al.*, 2003; Madhwani and McBain, 2011; Zaura *et al.*, 2011). Overall, it would appear the possibilities are vast and only a few could be mentioned here.

Since the anti-caries action of fluoride has been relatively well established, fluoride was the prime candidate for assessing the application of the CDFF for the investigation of caries preventative agents in general. It was demonstrated that at

0.05 ppm, fluoride was unable to exert a significant anti-caries effect despite being continuously present during and between sucrose exposures (Chapter 5). The current study emphasises that previous reports of fluoride efficacy as low as 0.03 ppm fluoride may have overestimated its activity since the influence of biofilm was not included in these pH cycling studies (Featherstone *et al.*, 1986; Featherstone *et al.*, 1990; Featherstone and Zero, 1992). Laminated lesions in 0.05 ppm fluoride exposed enamel demonstrate the role of the biofilm present, since these structures have previously been attributed to cycles of remineralisation and demineralisation in caries formation and the administration of fluoride (Silverstone, 1982; Schupbach *et al.*, 1990). It is likely that only some fluoride was able to penetrate the biofilm and fully exert its anti-caries effects on the underlying enamel, between each sucrose pulse. It is possible that fluoride was bound in the matrix of the biofilm (Rose *et al.*, 1996) and released when the system underwent each sucrose challenge. In this paradigm, coupled diffusion into the lesion would have followed and selective adsorption of fluoride would result in layers of enamel more resistant to acidic attack. The consequence of laminated lesions on the overall resistance of the lesion to further demineralisation is unclear from the present study and could be further investigated. In agreement with previous work, it appears laminated zones push the lesion front further into the depth of the affected enamel. Other studies indicate that regions of the lesion body behind laminated zones are successively more susceptible to acids (Lagerweij and ten Cate, 2006; ten Cate *et al.*, 2006) and that less fluoride was able to penetrate past the lamination body. Therefore, these lesions may be less easy to remineralise and in summary the findings of this study emphasise the importance of maintaining elevated levels of fluoride in the oral environment throughout the day. Significant deposits of fluoride in the form of calcium fluoride were observed on the surface of enamel beneath biofilm exposed to 1,450 ppm fluoride continuously (Chapter 5). It appears that these temporary stores of fluoride formed, given sufficient time, and remained despite likely acid production by the bacteria of the

biofilm. Although it is currently understood that the primary anti-caries action of fluoride regards its effect on enamel solubility, and in favouring remineralisation, the fluoride at this concentration also likely modulated lactic and acetic acid production by biofilm bacteria. No significant caries lesions and some surface loss was observed from enamel under these conditions but, this surface loss was deemed insignificant in clinical terms and is likely a consequence of the slight acidity of the non-buffered sodium fluoride solution in combination with any acids of bacterial origin. Furthermore, no surface loss or caries lesions were observed in enamel pulsed for two minutes with 1,450 ppm fluoride either twice or thrice daily and sucrose exposures at high frequency (Chapter 6). This confirmed the anti-caries efficacy of fluoride when supplied in frequent applications throughout the day. It may initially indicate that the addition of a third fluoride application did not explicitly improve the anti-caries capability of fluoride under these conditions. Even so, the reservoir of fluoride was greater within biofilm exposed thrice daily than twice daily, suggesting that over a longer period of time the benefit of the third application may be more distinguishable. This would be of value particularly in biofilm remaining at inaccessible sites of the dentition over prolonged periods of time. It would be interesting to elucidate whether more fluoride, if any, was retained in reservoir as calcium fluoride deposits on the enamel surfaces following conditions, such as in this experiment, simulating the daily use of fluoride dentifrices or washes in relatively short exposures. No caries lesions were observed in enamel exposed to 228 ppm fluoride either twice or thrice daily. However, the marked presence of red fluorescence within the enamel surfaces exposed twice daily compared to barely any in twice daily 1,450 ppm fluoride exposed enamel may be an indication of the reduced effects of fluoride on the metabolism of the biofilm bacteria at this lower concentration of fluoride.

A number of studies have demonstrated that oral microorganisms fluoresce when illuminated with the QLF light and have been associated with a number of fluorophores (Konig *et al.*, 1994). As caries progresses the increasing enamel porosity means the fluorescent metabolic products are able to increasingly infiltrate the enamel structure as seen in the present study. Not all microorganisms fluoresce red however; *S. mutans* for example reportedly exhibits green fluorescence (Konig *et al.*, 1993; Lennon *et al.*, 2006). Nonetheless, it appears that the red fluorescent abilities of caries important microorganisms, including *S. mutans* may be substrate inducible (Volgenant *et al.*, 2013). Therefore, so far, it can only be claimed that red fluorescence is likely related to the intrinsic metabolic products of the oral biofilm on a whole, and not inherently to the presence of the specific bacteria. Overall, the current study provided evidence of biofilm red fluorescence as an indicator of bacterial metabolism and biofilm maturation in line with the work of others (Kim, 2014; Bittar, 2014). Sucrose exposed biofilm emitted successively higher levels of red fluorescence while fluoride inhibited increases in red fluorescence over time. In this regard, the current study innovatively contributes to our understanding of the effects of fluoride on biofilm red fluorescence. It also revealed that bacteria may differentially exhibit proportions of both green and red fluorescence when in community and complex microbial interactions may affect biofilm fluorescence characteristics. Regarding the theory that biofilm red fluorescence can be related to its cariogenicity, the current findings reveal that red fluorescence (as R/G ratio) is not always well associated with factors of the caries disease process. Further investigation is required and the development of more appropriate statistical model approaches would be invaluable to this field of research. QLF-D appears to have potential as a device for plaque assessment, but evaluation should not simply focus on the notion of identifying quantities of specific causative microorganisms but concentrate on its use in indicating the metabolic activity of biofilms.

8.2 The use of TMR, QLF and NCSP in caries research

The current investigations provided insight into the application of QLF for quantifying caries in comparison to the current 'gold standard' techniques in mineralisation analyses, TMR. As discussed extensively (see Sections 3.4.2 and 5.4.2 specifically), the relationship between TMR and QLF values was strong even in this multispecies biological caries model. However, the strength of correlation was highly influenced by the procedures for generating sections of enamel for TMR analysis and was only good when TMR was optimal. It has been demonstrated numerously throughout this thesis that mineral loss and lesion depth can be highly variable across caries lesions produced in biological models for caries. TMR sections sometimes differed markedly despite originating from the same enamel lesion. Furthermore, since TMR involves analysis of single or multiple slices as a representation of the lesion whilst QLF quantifies the lesion on a whole, direct correlation is problematic. Some tissue loss is inevitable during the TMR procedure and in this way quantification by QLF has its advantages. Nonetheless, TMR remains an invaluable research tool since it allows us to obtain direct profiles of mineral loss and visualise the characteristics of the lesion, enabling researchers to confirm lesion types. Overall, the study provided further evidence for the validation of QLF as a tool to monitor caries *in vivo*. This technique is non-destructive and its application of the longitudinal measurement of de- and remineralisation has already been demonstrated. Finally, the current study is the first to explore the potential use of NCSP in enamel caries research. NCSP was able to distinguish prism-like structures at the enamel surface consistent with those found by SEM and AFM in other demineralisation studies; attributable to the preferential dissolution of the crystalline phase by acidic products of the biofilm bacterial metabolism causing the protein-rich inter-rod space to become more prominent. Nanometre scale surface loss was detected and corresponded to the TMR and QLF measures of caries progression.

Therefore, the application of this technique, in resolving subtle surface changes, is promising and worthy of further testing.

8.3 General evaluation of the CDFF biological caries model and scope for the study of other caries preventative agents

Regarding the relation of models to reality; although laboratory model systems exist on a spectrum beneath the direct study of a phenomenon in nature, the model developed here represents *in vivo* conditions relatively well and is more inclusive as a sophisticated model of caries than ones before it. The representative nature of biofilm produced in the current study is claimed based upon its microbiological composition and the outcome of representative caries lesions. Investigations such as visualising the structure of biofilm using techniques like SEM would confirm the similarity of the CDFF biofilm in this study to supragingival dental plaque. Nonetheless, these similarities have been reported in other CDFF biofilm studies with similar experimental design (Pratten, 1998; Matharu *et al.*, 2001). Furthermore, laboratory based models such as the CDFF, bear advantages when attempting to assess the efficacy of experimental products, since ethical considerations are far less than in clinical or *in situ* studies. The CDFF approach also enabled the standardisation of, often confounding, factors that can affect the results and interpretation of findings from this latter type of study. For example, it has traditionally been difficult to quantify the importance of associations between the use of anti-caries products and caries incidence. In reality there are a host of variables influencing caries risk and extricating the influence of the variable of interest can be increasingly complex. Randomised clinical trials would be the optimal mode of investigation, wherein subjects may be instructed to brush less frequently, not at all, or use experimental products with only suspected cariostatic effect. Considering our understanding of caries, this approach would be unethical.

In line with *in vivo* and *in vitro* work by others (Paes Leme *et al.*, 2004; Aires *et al.*, 2006; Paes Leme *et al.*, 2006), the current investigations demonstrated a dose-dependent response to sucrose concentration in enamel demineralisation over time, when multispecies biofilm were pulsed with sucrose at high frequency (Chapter 3). This provided part of the basis of the subsequent fluoride studies. Since significant variation has been reported in the past by fellow CDFF researchers, the variability of the current model system was investigated and motivated the further development of the CDFF model (Chapter 4) (Bakht *et al.*, 2010; Hope *et al.*, 2012). The novel dual model minimised the variability and degree of difference between CDFFs to be compared and consequently provided the basis of direct comparisons in the possible subtle effects of fluoride on biofilm bacteria and enamel. The screening of potential anti-caries agents using this approach holds considerable value since compounds and concentrations can be selected prior to expensive clinical trials. In this regard, promising avenues for future work would be the investigation of compounds, or their combinations, capable of improving the penetration of fluoride through biofilm and its delivery to enamel.

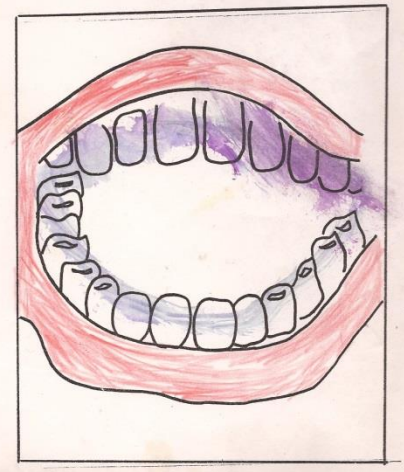
One area of particular interest would be to investigate the anti-caries potential of novel formulations of casein phosphopeptides (CPPs) and amorphous calcium phosphate (ACP) using the current model. It has been established that the ability of fluoride to promote remineralisation and its retention in plaque is limited by the availability of calcium and phosphate ions in the oral environment (Dorozhkin, 2007; Reynolds, 2008; Cochrane *et al.*, 2010). Calcium pre-rinsing enhanced fluoride retention in plaque when followed by a separate fluoride rinse (Vogel, 1997). The literature to date, suggests these vehicle molecules are particularly promising for the delivery of calcium and phosphate. Unstabilised ACP (Combes and Rey, 2010) has been effective in preventing caries progression, particularly in its fluoridated form

(ACFP). Evidence supports the action of rapidly formed ACP phases in remineralising subsurface lesions and yet there are concerns regarding the possible promotion of calculus formation due to the transformation of ACP compounds to poorly soluble phases in the mouth. Delivering ACP stabilised by CPP in the form of CPP-ACP/CPP-ACFP appear a promising approach to circumvent the potential for calculus with the use of ACPs alone. The approach of the current study could be expanded to investigate such queries as the residence time of CPP-AC(F)P, the bioactivity of CPP-AC(F)P as affected by different behaviours in food consumption, the comparative effect of the formation of unstabilised ACP, ACFP, CPP-ACP and CPP-ACFP on caries prevention. The QLF would be useful in the latter investigations since it is able to provide insight into whether calculus formation is promoted. It would also be interesting to investigate the impact of a combination of cariogenic compounds with the aim to model dietary consumption more representatively. For example, exposing the CDFF biofilm to various combinations of starch, sucrose, glucose and fructose and developing pulsing strategies of different durations and frequencies. This could model the complexity of the modern diet by taking into account more factors related to the increasing tendency towards combinations of grazing and snacking with a number of food items.

In conclusion, the results of this study demonstrated that the CDFF can be used to produce multispecies biofilm under conditions similar to those of the oral environment and investigate its cariogenicity in response to diet and anti-caries therapies.

name: Khush

teeth.



Check-up Time

Fill in the missing words.

My dentist helps me look after my teeth.

I visit my dentist for check-ups.

I have a check-up every six months.

I sit in a big chair that moves up and down.

The dentist uses a small, round mirror to look at my teeth.

months **chair** **dentist** **mirror** **check-ups**

Colour the picture.

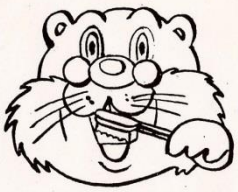
My Sparkling Smile

Use the poster to help you fill in the missing words.

6 It's easy to look after your teeth.

Follow these five steps to a sparkling smile.

1 I brush my teeth properly.



2 I use a Fluoride toothpaste.

3 I don't eat sweets too often.

4 I eat healthy snacks.

5 I visit my dentist for check-ups.

6 Colour the pictures.

References

Aas, J.A., Paster, B.J., Stokes, L.N., Olsen, I. and Dewhirst, F.E. (2005). "Defining the normal bacterial flora of the oral cavity." Journal of Clinical Microbiology **43**(11): 5721-5732.

Ablal, M.A., Kaur, J.S., Cooper, L., Jarad, F.D., Milosevic, A., Higham, S.M. and Preston, A.J. (2009). "The erosive potential of some alcopops using bovine enamel: an *in vitro* study." Journal of Dentistry **37**(11): 835-839.

Aires, C.P., Tabchoury, C.P.M., Del Bel Cury, A.A., Koo, H. and Cury, J.A. (2006). "Effect of sucrose concentration on dental biofilm formed *in situ* and on enamel demineralization." Caries Research **40**(1): 28-32.

Ajdic, D., McShan, W.M., McLaughlin, R.E., Savic, G., Chang, J., Carson, M.B., Primeaux, C., Tian, R.Y., Kenton, S., Jia, H.G., Lin, S.P., Qian, Y.D., Li, S.L., Zhu, H., Najar, F., Lai, H.S., White, J., Roe, B.A. and Ferretti, J.J. (2002). "Genome sequence of *Streptococcus mutans* UA159, a cariogenic dental pathogen." Proceedings of the National Academy of Sciences of the United States of America **99**(22): 14434-14439.

Ajdic, D. and Pham, V.T.T. (2007). "Global transcriptional analysis of *Streptococcus mutans* sugar transporters using microarrays." Journal of Bacteriology **14**: 5049-5059.

Ajdic, D. and Chen, Z. (2013). "A novel phosphotransferase system of *Streptococcus mutans* is responsible for transport of carbohydrates with α-1,3 linkage." Molecular Oral Microbiology **28**(2): 114-128.

Akizawa, Y., Sakurai, Y., Hara, N., Fujita, Y., Nagai, M., Nakamura, Y., Sakata, K. and Yanagawa, H. (1990). "An epidemiological study of the influence of sweets intake and toothbrushing on dental caries among children in Japan." Asia-Pacific Journal of Public Health **4**(4): 242-250.

Al-Hashimi, I. and Levine, M.J. (1989). "Characterization of *in vivo* salivary-derived enamel pellicle." Archives of Oral Biology **34**(4): 289-295.

Al-Khateeb, S., Oliveby, A., de Josselin de Jong, E. and Angmar-Mansson, B. (1997a). "Laser fluorescence quantification of remineralisation *in situ* of incipient enamel lesions: influence of fluoride supplements." Caries Research **31**(2): 132-140.

Al-Khateeb, S., ten Cate, J.M., Angmar-Månsson, B., de Josselin de Jong, E., Sundström, G., Exterkate, R.A. and Oliveby, A. (1997b). "Quantification of formation and remineralization of artificial enamel lesions with a new portable fluorescence device." Advances in Dental Research **11**(4): 502-506.

Al-Khateeb, S., Exterkate, R., Angmar-Mansson, B. and ten Cate, B. (2000). "Effect of acid-etching on remineralization of enamel white spot lesions." Acta Odontologica Scandinavica **58**(1): 31-36.

Al-Radha, A.S.D., Younes, C., Diab, B.S. and Jenkinson, H.F. (2013). "Essential oils and zirconia dental implant materials." International Journal of Oral & Maxillofacial Implants **28**(6): 1497-1505.

Alfano, R.R. and Yao, S.S. (1981). "Human teeth with and without dental caries studied by visible luminescent spectroscopy." Journal of Dental Research **60**(2): 120-122.

Allan, I., Newman, H. and Wilson, M. (2002). "Particulate Bioglass (R) reduces the viability of bacterial biofilms formed on its surface in an *in vitro* model." Clinical Oral Implants Research **13**(1): 53-58.

Allesen-Holm, M., Barken, K.B., Yang, L., Klausen, M., Webb, J.S., Kjelleberg, S., Molin, S., Givskov, M. and Tolker-Nielsen, T. (2006). "A characterization of DNA release in *Pseudomonas aeruginosa* cultures and biofilms." Molecular Microbiology **59**(4): 1114-1128.

Altman, D.G. (1991). "Statistical analysis of comparison between laboratory methods." Journal of Clinical Pathology **44**(8): 700-701.

Amaechi, B.T. and Higham, S.M. (2002). "Quantitative light-induced fluorescence: a potential tool for general dental assessment." Journal of Biomedical Optics **7**(1): 7-13.

Ando, M., Hall, A.F., Schemehorn, B.R., Stookey, G.K., Analoui, M. and Eckert, G.J. (1997). "Relative ability of laser fluorescence techniques to quantitate early mineral loss *in vitro*." Caries Research **31**(2): 125-131.

Ando, M., Analoui, M., Schemehorn, B. and Stookey, G. (1999). Comparison of light-induced and laser-induced fluorescence methods for the detection and quantification of enamel demineralization. Proceedings of Lasers in Dentistry V. Featherstone, J.D.B., Rechmann, P. and Fried, D. **3593:** 148-153.

Ando, M., van der Veen, M.H., Schemehorn, B.R. and Stookey, G.K. (2001). "Comparative study to quantify demineralized enamel in deciduous and permanent teeth using laser- and light-induced fluorescence techniques." Caries Research **35**(6): 464-470.

Ando, M., Schemehom, B.R., Eckert, G.J., Zero, D.T. and Stookey, G.K. (2003). "Influence of enamel thickness on quantification of mineral loss in enamel using laser-induced fluorescence." Caries Research **37**(1): 24-28.

Angmar-Månsson, B., Al-Khateeb, S. and Tranaeus, S. (1996). Intraoral use of quantitative light-induced fluorescence for caries detection. Early Detection of Dental Caries: Proceedings of the 1st Annual Indiana Conference. Stookey, G.K. Indianapolis, Indiana, Indiana University**:** 105-118.

Angmar-Månsson, B. and ten Bosch, J.J. (2001). "Quantitative light-induced fluorescence (QLF): a method for assessment of incipient caries lesions." Dentomaxillofacial Radiology **30**(6): 298-307.

Angmar, B., Carlström, D. and Glas, J.E. (1963). "Studies on the ultrastructure of dental enamel: IV. The mineralization of normal human enamel." Journal of Ultrastructure Research **8**(1–2): 12-23.

Anwar, H., van Biesen, T., Dasgupta, M., Lam, K. and Costerton, J.W. (1989). "Interaction of biofilm bacteria with antibiotics in a novel *in vitro* chemostat system." Antimicrobial Agents and Chemotherapy **33**(10): 1824-1826.

Aoki, H., Shiroza, T., Hayakawa, M., Sato, S. and Kuramitsu, H.K. (1986). "Cloning of a *Streptococcus mutans* glucosyltransferase gene coding for insoluble glucan synthesis." Infection and Immunity **53**(3): 587-594.

Arends, J. and Schuthof, J. (1980). "Microhardness and lesion depth studies of artificial caries lesions - a comparison of gelatin and HEC based systems." Journal De Biologie Buccale **8**(2): 175-181.

Arends, J., Schuthof, J. and Jongebloed, W.G. (1980). "Lesion depth and microhardness indentations on artificial white spot lesions." Caries Research **14**(4): 190-195.

Arends, J., Christoffersen, J., Christoffersen, M.R. and Schuthof, J. (1983a). "Influence of fluoride concentration on the progress of demineralisation in bovine enamel at pH 4.5." Caries Research **17**(5): 455-457.

Arends, J., Gelhard, T., Lodding, A. and Odelius, H. (1983b). "Fluoride gradients in enamel lesions after *in vivo* remineralisation period of 3 months - a SIMS study." Caries Research **17**(2): 169-170.

Arends, J., Ogaard, B., Ekstrand, J. and Rolla, G. (1984). "Experimental enamel caries in man - a microradiographic investigation." Journal of Dental Research **63**: 255-255.

Arends, J. and Christoffersen, J. (1986). "The nature of early caries lesions in enamel." Journal of Dental Research **65**(1): 2-11.

Arends, J. and Christoffersen, J. (1990). "Nature and role of loosely bound fluoride in dental caries." Journal of Dental Research **69 Spec No**: 601-605; discussion 634-606.

Arends, J., de Vries, J. and Ruben, J. (1992). "The effect of an experimental mouthrinse on enamel lesion remineralization *in vitro*." Journal De Biologie Buccale **20**(1): 63-66.

Argenta, R.M.O., Tabchoury, C.P.M. and Cury, J.A. (2003). "A modified pH-cycling model to evaluate fluoride effect on enamel demineralization." Brazilian Oral Research **17**(3): 241-246.

Armstrong, W.G. (1963). "Fluorescence characteristics of sound and carious human dentine preparations." Archives of Oral Biology **8**(2): 79-90.

Arweiler, N., Netuschil, L., Beier, D., Grunert, S., Heumann, C., Altenburger, M., Sculean, A., Nagy, K., Al-Ahmad, A. and Auschill, T. (2014). "Action of food preservatives on 14-days dental biofilm formation, biofilm vitality and biofilm-derived enamel demineralisation *in situ*." Clinical Oral Investigations **18**(3): 829-838.

Ashley, F.P. and Wilson, R.F. (1977a). "Dental plaque and caries. A 3-year longitudinal study in children." British Dental Journal **142**(3): 85-91.

Ashley, F.P. and Wilson, R.F. (1977b). "The relationship between dietary sugar experience and the quantity and biochemical composition of dental plaque in man." Archives of Oral Biology **22**(7): 409-414.

Ashley, P.F., Attrill, D.C., Ellwood, R.P., Worthington, H.V. and Davies, R.M. (1999). "Toothbrushing habits and caries experience." Caries Research **33**(5): 401-402.

Ashmore, H., Wilson, S.J. and Vanabbe, N.J. (1972). "Measurement *in vitro* of dentin abrasion by toothpaste." British Dental Journal **133**(2): 60-66.

Atkinson, B. and Fowler, H.W. (1974). The significance of microbial film in fermenters. Advances in Biochemical Engineering, Volume 3. Ghose, T.K., Fiechter, A. and Blakebrough, N., Springer Berlin Heidelberg. **3:** 221-277.

Auschill, T.M., Artweiler, N.B., Netuschil, L., Brecx, M., Reich, E. and Sculean, A. (2001). "Spatial distribution of vital and dead microorganisms in dental biofilms." Archives of Oral Biology **46**(5): 471-476.

Backer Dirks, C. (1966). "Post eruptive changes in enamel." Journal of Dental Research **45**: 503-511.

Baelum, V., van Palenstein Helderman, W., Hugoson, R., Yee, R. and Fejerskov, O. (2008). 'for richer, for poorer, in sickness and in health...' The role of dentistry in controlling caries. Dental Caries: the Disease and its Clinical Management. Fejerskov, O. and Kidd, E.A.M. Oxford, Wiley-Blackwell**:** 575-606.

Baier, R.E. (1984). Initial events in microbial film formation. Marine Biodetermination: an Interdisciplinary Approach. Costlow, J.D., Tipper, R.C. London, E & FN Spon**:** 57-62.

Bakht, K., Higham, S.M., de Josselin de Jong, E., Martin, G.C., Burnett, G. and Hope, C.K. (2010). "Novel constant-depth film fermenter model for oral biofilm development. 88th General Session & Exhibition of the IADR (abstract 1775)." Journal of Dental Research.

Baysan, A., Lynch, E., Ellwood, R., Davies, R., Petersson, L. and Borsboom, P. (2001). "Reversal of primary root caries using dentifrices containing 5,000 and 1,100 ppm fluoride." Caries Research **35**(1): 41-46.

Beighton, D. (2005). "The complex oral microflora of high-risk individuals and groups and its role in the caries process." Community Dentistry And Oral Epidemiology **33**(4): 248-255.

Belli, W.A., Buckley, D.H. and Marquis, R.E. (1995). "Weak acid effects and fluoride inhibition of glycolysis by *Streptococcus mutans* GS-5." Canadian Journal of Microbiology **41**(9): 785-791.

Bender, G.R., Sutton, S.V. and Marquis, R.E. (1986). "Acid tolerance, proton permeabilities, and membrane ATPases of oral streptococci." Infection and Immunity **53**(2): 331-338.

Bender, G.R. and Marquis, R.E. (1987). "Membrane ATPases and acid tolerance of *Actinomyces viscosus* and *Lactobacillus casei*." Applied and Environmental Microbiology **53**(9): 2124-2128.

Benedict, H.C. (1928). "A note on the fluorescence of teeth in ultra-violet rays." Science **67**(1739): 442.

Benson, P.E., Pender, N. and Higham, S.M. (2003a). "Quantifying enamel demineralization from teeth with orthodontic brackets - a comparison of two methods. Part 2: validity." European Journal of Orthodontics **25**(2): 159-165.

Benson, P.E., Pender, N. and Higham, S.M. (2003b). "Quantifying enamel demineralization from teeth with orthodontic brackets - a comparison of two methods. Part 1: repeatability and agreement." European Journal of Orthodontics **25**(2): 149-158.

Bergman, G. and Lind, P.O. (1966). "A quantitative microradiographic study of incipient enamel caries." Journal of Dental Research **45**(5P2): 1477-1484.

Bernardin, J.D., Mudawar, I., Walsh, C.B. and Franses, E.I. (1997). "Contact angle temperature dependence for water droplets on practical aluminum surfaces." International Journal of Heat and Mass Transfer **40**(5): 1017-1033.

Bibby, B.G. and van Kesteren, M. (1940). "The effect of fluorine on mouth bacteria." Journal of Dental Research **19**: 391-402.

Bibby, B.G., Gustafson, G. and Davies, G.N. (1958). "A critique of three theories of caries attack." International Dental Journal **8**: 685-695.

Binnie, W.H. and Mitchell, D.F. (1973). "Induced calcification in subdermal tissues of rat." Journal of Dental Research **52**(5): 1087-1091.

Birkhed, D., Edwardsson, S., Svensson, B., Moskovitz, F. and Frostell, G. (1978). "Acid production from sorbitol in human dental plaque." Archives of Oral Biology **23**(11): 971-975.

Bittar, D.G., Pontes, L.R.A., Calvo, A.F.B., Novaes, T.F., Braga, M.M., Freitas, P.M., Tabchoury, C.P.M. and Mendes, F.M. (2014). "Is the red fluorescence of dental plaque related to its cariogenicity?" Journal of Biomedical Optics **19**(6): 065004.065001-065004.065006.

Bjelkhagen, H., Sundstrom, F., Angmar-Mansson, B. and Ryden, H. (1982). "Early detection of enamel caries by the luminscence excited by visible laser-light." Swedish Dental Journal **6**(1): 1-7.

Black, G.V. (1884). The formation of poisons by micro-organisms. A biological study of the germ theory of disease. Philadelphia, P. Blakiston, son & co.

Bland, J.M. and Altman, D.G. (1986). "Statistical methods for assessing agreement between two methods of clinical measurement." The Lancet **327**(8476): 307-310.

Bland, J.M. and Altman, D.G. (1999). "Measuring agreement in method comparison studies." Statistical Methods in Medical Research **8**(2): 135-160.

BLAST. (assession no. AEO14133). from http://www.genome.ou.edu/smutans.html.

Boote, D.N. and Beile, P. (2005). "Scholars Before Researchers: On the Centrality of the Dissertation Literature Review in Research Preparation." Educational Researcher **34**(6): 3-15.

Borei, H. (1945). "Inhibition of cellular oxidation by fluoride." Arkiv for Kemi Mineralogi Och Geologi **20**(2-3): 1-214.

Borsboom, P.C., vd Mei, H.C. and Arends, J. (1985). "Enamel lesion formation with and without 0.12 ppm F in solution." Caries Research **19**(5): 396-402.

Bowden, G.H.W., Odlum, O., Nolette, N. and Hamilton, I.R. (1982). "Microbial populations growing in the presence of fluoride at low pH isolated from dental plaque of children living in an area with fluoridated water." Infection and Immunity **36**(1): 247-254.

Bowen, W.H. and Hewitt, M.J. (1974). "Effect of fluoride on extracellular polysaccharide production by *Streptococcus mutans*." Journal of Dental Research **53**(3): 627-629.

Bowen, W.H. (2002). "Do we need to be concerned about dental caries in the coming millennium?" Critical Reviews in Oral Biology & Medicine **13**(2): 126-131.

Bowen, W.H. and Koo, H. (2011). "Biology of *Streptococcus mutans*-derived glucosyltransferases: role in extracellular matrix formation of cariogenic biofilms." Caries Research **45**(1): 69-86.

Boyde, A. and Lester, K.S. (1967). "Electron microscopy of resorbing surfaces of dental hard tissues." Zeitschrift Fur Zellforschung Und Mikroskopische Anatomie **83**(4): 538-548.

Boyde, A. and Jones, S.J. (1996). "Scanning electron microscopy of bone: Instrument, specimen, and issues." Microscopy Research and Technique **33**(2): 92-120.

Bradshaw, D.J., McKee, A.S. and Marsh, P.D. (1989). "Effects of carbohydrate pulses and pH on population shifts within oral microbial communities *in vitro*." Journal of Dental Research **68**(9): 1298-1302.

Bradshaw, D.J., Homer, K.A., Marsh, P.D. and Beighton, D. (1994). "Metabolic cooperation in oral microbial communities during growth on mucin." Microbiology **140**(12): 3407-3412.

Bradshaw, D.J., Marsh, P.D., Allison, C. and Schilling, K.M. (1996a). "Effect of oxygen, inoculum composition and flow rate on development of mixed-culture oral biofilms." Microbiology **142**(3): 623-629.

Bradshaw, D.J., Marsh, P.D., Schilling, K.M. and Cummins, D. (1996b). "A modified chemostat system to study the ecology of oral biofilms." Journal of Applied Microbiology **80**(2): 124-130.

Branda, S.S., Vik, A., Friedman, L. and Kolter, R. (2005). "Biofilms: the matrix revisited." Trends in Microbiology **13**(1): 20-26.

Bratthall, D., HanselPetersson, G. and Sundberg, H. (1996). "Reasons for the caries decline: What do the experts believe?" European Journal of Oral Sciences **104**(4): 416-422.

Brazier, J.S. (1986). "A note on ultra-violet red fluorescence of anaerobic bacteria *in vitro*." Journal of Applied Bacteriology **60**(2): 121-126.

Broukal, Z. and Zajicek, O. (1974). "Amount and distribution of extracellular polysaccharides in dental microbial plaque." Caries Research **8**(2): 97-104.

Brown, C.M., Ellwood, D.C. and Hunter, J.R. (1977). "Growth of bacteria at surfaces - influence of nutrient limitation." FEMS Microbiology Letters **1**(3): 163-166.

Brown, L.R., White, J.O., Horton, I.M., Perkins, D.H., Streckfuss, J.L. and Dreizen, S. (1981). "Effects of a single application of sodium fluoride gel on dental plaque acidogenesis." Journal of Dental Research **60**(8): 1396-1402.

Brown, L.R., White, J.O., Horton, I.M., Dreizen, S. and Streckfuss, J.L. (1983). "Effect of continuous fluoride gel use on plaque fluoride retention and microbial activity." Journal of Dental Research **62**(6): 746-751.

Brudevold, F. (1974). Fluoride. Improving dental practice through preventive measures. Bernier, J.L. and Muhler, J.C. St Louis, Mosby**:** 77-103.

Brudevold, F., Goulet, D., Attarzadeh, F. and Tehrani, A. (1988). "Demineralization potential of different concentrations of gelatinized wheat starch." Caries Research **22**(4): 204-209.

Bruun, C., Givskov, H. and Thylstrup, A. (1984). "Whole saliva fluoride after toothbrushing with NaF and MFP dentifrices with different F concentrations." Caries Research **18**(3): 282-288.

Bruun, C., Qvist, V. and Thylstrup, A. (1987). "Effect of flavour and detergent on fluoride availability in whole saliva after use of NaF and MFP dentifrices." Caries Research **21**(5): 427-434.

Bryers, J.D. (1984). "Biofilm formation and chemostat dynamics: pure and mixed culture considerations." Biotechnology and Bioengineering **26**(8): 948-958.

Bullitt, E. and Makowski, L. (1995). "Structural polymorphism of bacterial adhesion pili." Nature **373**: 164-167.

Burne, R.A., Chen, Y.-Y.M. and Penders, J.E.C. (1997). "Analysis of gene expression in *Streptococcus mutans* in biofilms *in vitro*." Advances in Dental Research **11**(1): 100-109.

Burt, B.A., Eklund, S.A. and Loesche, W.J. (1986). "Dental benefits of limited exposure to fluoridated water in childhood." Journal of Dental Research **65**(11): 1322-1325.

Burt, B.A. (1993). "Relative consumption of sucrose and other sugars - has it been a factor in reduced caries experience?" Caries Research **27**: 56-63.

Busscher, H.J., Cowan, M.M. and van der Mei, H.C. (1992). "On the relative importance of specific and non-specific approaches to oral microbial adhesion." FEMS Microbiology Letters **88**(3-4): 199-210.

Buzalaf, M.A.R., Pessan, J.P., Honorio, H.M. and ten Cate, J.M. (2011). Mechanisms of action of fluoride for caries control. Fluoride and the Oral Environment. Buzalaf, M.A.R. Basel; Switzerland, S Karger AG**:** 97-114.

Bystrom, A., Claesson, R. and Sundqvist, G. (1985). "The antibacterial effect of camphorated P monochlorophenol, camphorated phenol and calcium hydroxide in the treatment of infected root canals." Endodontics and Dental Traumatology **1**(5): 170-175.

Carlsson, J. and Griffith, C.J. (1974). "Fermentation products and bacterial yields in glucose-limited and nitrogen-limited cultures of streptococci." Archives of Oral Biology **19**(12): 1105-1109.

Carter, W.J., Dunn, J.K., Fosdick, L.S. and Moore, B.W. (1956). "The formation of lactic acid in dental plaques. I. Caries-active individuals." Journal of Dental Research **35**(5): 778-785.

Ccahuana-Vásquez, R.A., Tabchoury, C.P.M., Tenuta, L.M.A., Del Bel Cury, A.A., Vale, G.C. and Cury, J.A. (2007). "Effect of frequency of sucrose exposure on dental biofilm composition and enamel demineralization in the presence of fluoride." Caries Research **41**(1): 9-15.

Cenci, M.S., Pereira-Cenci, T., Cury, J.A. and ten Cate, J.M. (2009). "Relationship between gap size and dentine secondary caries formation assessed in a microcosm biofilm model." Caries Research **43**(2): 97-102.

Characklis, W.G. (1973). "Attached microbial growth 2. Frictional resistance due to microbial slimes." Water Research **7**(9): 1249-1258.

Characklis, W.G. (1981). "Fouling biofilm development - a process analysis." Biotechnology and Bioengineering **23**(9): 1923-1960.

Characklis, W.G. and Cooksey, K.E. (1983). "Biofilms and microbial fouling." Advances in Applied Microbiology **29**: 93-138.

Chen, X., Jiang, Z.H., Chen, S.F. and Qin, W.S. (2010). "Microbial and bioconversion production of D-xylitol and its detection and application." International Journal of Biological Sciences **6**(7): 834-844.

Chen, Y.Y.M., Clancy, K.A. and Burne, R.A. (1996). "*Streptococcus salivarius* urease: genetic and biochemical characterization and expression in a dental plaque *Streptococcus*." Infection and Immunity **64**(2): 585-592.

Chernish, R.N. and Aaron, S.D. (2003). "Approach to resistant gram-negative bacterial pulmonary infections in patients with cystic fibrosis." Current Opinion in Pulmonary Medicine **9**(6): 509-515.

Chestnutt, I.G., Schafer, F., Jacobson, A.P.M. and Stephen, K.W. (1998). "The influence of toothbrushing frequency and post-brushing rinsing on caries experience in a caries clinical trial." Community Dentistry and Oral Epidemiology **26**(6): 406-411.

Cholodny Kiew, N. (1930). "Über eine neue methode zur untersuchung der bodenmikroflora." Archiv für Mikrobiologie **1**(1): 620-652.

Christensen, B.E. (1989). "The role of extracellular polysaccharides in biofilms." Journal of Biotechnology **10**(3–4): 181-202.

Cisar, J.O., Barsumian, E.L., Curl, S.H., Vatter, A.E., Sandberg, A.L. and Siraganian, R.P. (1981). "Detection and localisation of a lectin on *Actinomyces viscosus* T14V by monoclonal antibodies." Journal of Immunology **127**(4): 1318-1322.

Clancy, K.A., Pearson, S., Bowen, W.H. and Burne, R.A. (2000). "Characterization of recombinant, ureolytic *Streptococcus mutans* demonstrates an inverse relationship between dental plaque ureolytic capacity and cariogenicity." Infection and Immunity **68**(5): 2621-2629.

Clarkson, B.H., Wefel, J.S. and Silverstone, L.M. (1981). "Redistribution of enamel fluoride during white spot lesion formation - an *in vitro* study on human dental enamel." Caries Research **15**(2): 158-165.

Cochrane, N.J., Cai, F., Huq, N.L., Burrow, M.F. and Reynolds, E.C. (2010). "New approaches to enhanced remineralization of tooth enamel." Journal of Dental Research **89**(11): 1187-1197.

Cody, W.L., Wilson, J.W., Hendrixson, D.R., McIver, K.S., Hagman, K.E., Ott, C.M., Nickerson, C.A. and Schurr, M.J. (2008). "Skim milk enhances the preservation of thawed - 80 °C bacterial stocks." Journal of Microbiological Methods **75**(1): 135-138.

Cohen, J., Featherstone, J.D.B., Le, C.Q., Steinberg, D. and Feuerstein, O. (2014). "Effects of CO_2 laser irradiation on tooth enamel coated with biofilm." Lasers in Surgery and Medicine **46**(3): 216-223.

Combes, C. and Rey, C. (2010). "Review: amorphous calcium phosphates: synthesis, properties and uses in biomaterials." Acta Biomaterialia **6**: 3362-3378.

Connell, J.H. and Slatyer, R.O. (1977). "Mechanisms of succession in natural communities and their role in community stability and organisation." American Naturalist **111**(982): 1119-1144.

Coombe, R.A., Tatevossian, A. and Wimpenny, J.W.T. (1981). Bacterial thin films as *in vitro* models for dental plaque. Surface and Colloid Phenomena in the Oral Cavity: Methodological Aspects. Frank, R. and Leach, S. London, Information Retrieval**:** 239-249.

Coombe, R.A., Tatevossian, A. and Wimpenny, J.W.T. (1982). An *in vitro* model for dental plaque (abstract). 30th Meeting, British Division of the International Association for Dental Research. University of Edinburgh, Scotland.

Coombe, R.A. (1984). Factors affecting the growth of thin bacterial films *in vitro*. Bacterial Adhesion and Preventive Dentistry. ten Cate, J.M., Leach, S.A. and Arends, J. Oxford, IRL Press**:** 193.

Copper, M., Batchelor, S.M. and Prosser, J.I. (1995). Is cell density signalling applicable to biofilms? The Life and Death of Biofilm. Wimpenny, J., Handley, P., Gilbert, P. and Lappin-Scott, H. Cardiff, Bioline Press**:** 93–97.

Cornelius, C.E. and Ludwig, G.D. (1967). "Red fluorescence of comedones - production of porphyrins by *Corynebacterium acnes*." Journal of Investigative Dermatology **49**(4): 368-370.

Corpe, W.A. (1970). "An acid polysaccharide produced by a primary film forming marine bacterium." Developments in Industrial Microbiology **11**: 402-412.

Costerton, J.W., Geesey, G.G. and Cheng, K.J. (1978). "How bacteria stick." Scientific American **238**(1): 86-95.

Costerton, J.W., Irvin, R.T. and Cheng, K.J. (1981). "The bacterial glycocalyx in nature and disease." Annual Review of Microbiology **35**: 299-324.

Costerton, J.W., Cheng, K.J., Geesey, G.G., Ladd, T.I., Nickel, J.C., Dasgupta, M. and Marrie, T.J. (1987). "Bacterial biofilms in nature and disease." Annual Review of Microbiology **41**: 435-464.

Costerton, J.W., Lewandowski, Z., Caldwell, D.E., Korber, D.R. and Lappin-Scott, H.M. (1995). "Microbial biofilms." Annual Review of Microbiology **49**(1): 711-745.

Costerton, J.W., Stewart, P.S. and Greenberg, E.P. (1999). "Bacterial biofilms: a common cause of persistent infections." Science **284**(5418): 1318-1322.

Cotti, E., Dessi, C., Piras, A. and Mercuro, G. (2011). "Can a chronic dental infection be considered a cause of cardiovascular disease? A review of the literature." International Journal of Cardiology **148**(1): 4-10.

Coulthwaite, L., Pretty, I.A., Smith, P.W., Higham, S.M. and Verran, J. (2006). "The microbiological origin of fluorescence observed in plaque on dentures during QLF analysis." Caries Research **40**(2): 112-116.

Creed, D. (1984a). "The photophysics and photochemistry of the near-UV absorbing amino-acids. 2. tyrosine and its simple derivatives." Photochemistry and Photobiology **39**(4): 563-575.

Creed, D. (1984b). "The photophysics and photochemistry of the near-UV absorbing amino-acids. 1. tryptophan and its simple derivatives." Photochemistry and Photobiology **39**(4): 537-562.

Cross, S.E., Kreth, J., Wali, R.P., Sullivan, R., Shi, W. and Gimzewski, J.K. (2009). "Evaluation of bacteria-induced enamel demineralization using optical profilometry." Dental Materials **25**(12): 1517-1526.

Curran, T.M., Lieou, J. and Marquis, R.E. (1995). "Arginine deiminase system and acid adaptation of oral streptococci." Applied and Environmental Microbiology **61**(12): 4494-4496.

Curran, T.M., Ma, Y., Rutherford, G.C. and Marquis, R.E. (1998). "Turning on and turning off the arginine deiminase system in oral streptococci." Canadian Journal of Microbiology **44**(11): 1078-1085.

Curtis, M.A. and Eastoe, J.E. (1978). "Comparison of free amino acid pools in dental plaque fluid from monkeys (*Macaca fascicularis*) fed on high and low sugar diets." Archives of Oral Biology **23**(11): 989-992.

Curtis, M.A., Kemp, C.W., Robrish, S.A. and Bowen, W.H. (1983). "Stickland reactions of dental plaque." Infection and Immunity **42**(1): 431-433.

Cury, J.A., Rebello, M.A. and Del Bel Cury, A.A. (1997). "*In situ* relationship between sucrose exposure and the composition of dental plaque." Caries Research **31**(5): 356-360.

Cury, J.A., Rebelo, M.A., Del Bel Cury, A.A., Derbyshire, M.T. and Tabchoury, C.P. (2000). "Biochemical composition and cariogenicity of dental plaque formed in the presence of sucrose or glucose and fructose." Caries Research **34**(6): 491-497.

Cvitkovitch, D.G. (2001). "Genetic competence and transformation in oral streptococci." Critical Reviews in Oral Biology & Medicine **12**(3): 217-243.

Daculsi, G., Menanteau, J., Kerebel, L.M. and Mitre, D. (1984). "Length and shape of enamel crystals." Calcified Tissue International **36**(5): 550-555.

Dalwai, F., Spratt, D.A. and Pratten, J. (2006). "Modeling shifts in microbial populations associated with health or disease." Applied and Environmental Microbiology **72**(5): 3678-3684.

Damato, F.A., Strang, R. and Stephen, K.W. (1988). "Comparison of solution-prepared and gel-prepared enamel lesions - an *in vitro* pH-cycling study." Journal of Dental Research **67**(8): 1122-1125.

Damen, J.J.M., Buijs, M.J. and ten Cate, J.M. (2002). "Acidogenicity of buccal plaque after a single rinse with amine fluoride - stannous fluoride mouthrinse solution." Caries Research **36**(1): 53-57.

Davies, D.G., Chakrabarty, A.M. and Geesey, G.G. (1993). "Exopolysaccharide production in biofilms: substratum activation of alginate gene expression by *Pseudomonas aeruginosa*." Applied and Environmental Microbiology **59**(4): 1181-1186.

De Boever, J., Hirzel, H.C. and Muhlemann, H.R. (1969). "The effect of concentrated sucrose solutions on pH of interproximal plaque." Helvetica Odontologica Acta **13**(1): 27-28.

de Boever, J. and Muhlemann, H.R. (1969). "pH of interproximal plaque with regard to continuous sucrose application." Helvetica Odontologica Acta **13**(2): 97-99.

de Josselin de Jong, E. and ten Bosch, J.J. (1985). "Error analysis of the microradiographic determination of mineral content in mineralised tissue slices." Physics in Medicine and Biology **30**(10): 1067-1075.

de Josselin de Jong, E., ten Bosch, J.J. and Noordmans, J. (1987). "Optimised microcomputer-guided quantitative microradiography on dental mineralised tissue slices." Physics in Medicine and Biology **32**(7): 887-899.

de Josselin de Jong, E., Sundstrom, F., Westerling, H., Tranaeus, S., ten Bosch, J.J. and Angmar-Mansson, B. (1995). "A new method for *in vivo* quantification of changes in initial enamel caries with laser fluorescence." Caries Research **29**(1): 2-7.

de Josselin de Jong, E.D., Higham, S.M., Smith, P.W., van Daelen, C.J. and van der Veen, M.H. (2009). "Quantified light-induced fluorescence, review of a diagnostic tool in prevention of oral disease." Journal of Applied Physics **105**(10): 7.

de la Fuente, R., Schleifer, K.H., Götz, F. and Köst, H.P. (1986). "Accumulation of porphyrins and pyrrole pigments by *Staphylococcus aureus* spp. anaerobius and its aerobic mutant." FEMS Microbiology Letters **35**(2-3): 183-188.

de Stoppelaar, J.D., van Houte, J. and Backer Dirks, O. (1970). "The effect of carbohydrate restriction on the presence of *Streptococcus mutans, Streptococcus sanguis* and iodophilic polysaccharide-producing bacteria in human dental plaque." Caries Research **4**(2): 114-123.

Deng, D.M., Buijs, M.J. and ten Cate, J.M. (2004). "The effects of substratum on the pH response of *Streptococcus mutans* biofilms and on the susceptibility to 0.2% chlorhexidine." European Journal of Oral Sciences **112**(1): 42-47.

Deng, D.M. and ten Cate, J.M. (2004). "Demineralization of dentin by *Streptococcus mutans* biofilms grown in the constant depth film fermentor." Caries Research **38**(1): 54-61.

Deng, D.M., van Loveren, C. and ten Cate, J.M. (2005). "Caries-preventive agents induce remineralization of dentin in a biofilm model." Caries Research **39**(3): 216-223.

Dennis, D.A., Gawronski, T.H., Sudo, S.Z., Harris, R.S. and Folke, L.E. (1975). "Variations in microbial and biochemical components of four-day plaque during a four-week controlled diet period." Journal of Dental Research **54**(4): 716-722.

Dewitte, K., Fierens, C., Stockl, D. and Thienpont, L.M. (2002). "Application of the Bland-Altman plot for interpretation of method-comparison studies: a critical investigation of its practice." Clinical Chemistry **48**(5): 799-801.

Dibdin, G.H., Shellis, R.P. and Wilson, C.M. (1976). "An apparatus for the continuous culture of micro-organisms on solid surfaces with special reference to dental plaque." Journal of Applied Bacteriology **40**(3): 261-268.

Dibdin, G.H., Wilson, C.M. and Shellis, R.P. (1983). "Effect of packing density and polysaccharide to protein ratio of plaque samples cultured *in vitro* upon their permeability." Caries Research **17**(1): 52-58.

Dibdin, G.H. and Shellis, R.P. (1988). "Physical and biochemical studies of *Streptococcus mutans* sediments suggest new factors linking the cariogenicity of plaque with its extracellular polysaccharide content." Journal of Dental Research **67**(6): 890-895.

Dills, S.S., Apperson, A., Schmidt, M.R. and Saier, M.H. (1980). "Carbohydrate transport in bacteria." Microbiological Reviews **44**(3): 385-418.

Distler, W. and Kröncke, A. (1980). "Acid formation by mixed cultures of cariogenic strains of *Streptococcus mutans* and *Veillonella alcalescens*." Archives of Oral Biology **25**(10): 655-658.

Distler, W. and Kroncke, A. (1983). "The acid pattern in human dental plaque." Journal of Dental Research **62**(2): 87-91.

Dobell, C. (1932). Antony van Leeuwenhoek and His 'Little Animals': being some account of the father of protozoology and bacteriology and his multifarious discoveries in these disciplines. Great Britain, Harcourt, Beace & Company.

Dodds, M.W.J. and Edgar, W.M. (1988). "The relationship between plaque pH, plaque acid anion profiles, and oral carbohydrate retention after ingestion of several 'reference foods' by human subjects." Journal of Dental Research **67**(5): 861-865.

Dolowy, W.C., Brandes, M.L., Gouterman, M., Parker, J.D. and Lind, J. (1995). "Fluorescence of dental calculus from cats, dogs, and humans and of bacteria cultured from dental calculus." Journal of Veterinary Dentistry **12**(3): 105-109.

Donlan, R.M. (2001). "Biofilms and device-associated infections." Emerging Infectious Diseases **7**(2): 277-281.

Donlan, R.M. and Costerton, J.W. (2002). "Biofilms: survival mechanisms of clinically relevant microorganisms." Clinical Microbiology Reviews **15**(2): 167-193.

Dorozhkin, S.V. (2007). "Calcium orthophosphates." Journal of Materials Science **42**(4): 1061-1095.

Douglas, C.W.I., Pease, A.A. and Whiley, R.A. (1990). "Amylase-binding as a discriminator among oral streptococci." FEMS Microbiology Letters **66**(1-3): 193-197.

Douglas, L.J. (2003). "*Candida* biofilms and their role in infection." Trends in Microbiology **11**(1): 30-36.

Douglass, C.W. and Day, J.M. (1979). "Cost and payment of dental services in the United States." Journal of Dental Education **43**(7): 330-348.

Downer, M.C. (1999). "Caries experience and sucrose availability: an analysis of the relationship in the United Kingdom over fifty years." Community Dental Health **16**(1): 18-21.

Driessens, F.C. (1982). "Mineral aspects of dentistry." Monographs in Oral Science **10**: 1-215.

Driessens, F.C.M., Theuns, H.M., Borggreven, J. and Heijligers, H.J.M. (1987). "Electron-microprobe analysis and microradiography of some artificial laminated carious lesions." Caries Research **21**(3): 222-227.

Drucker, D.B. and Melville, T.H. (1968). "Fermentation end-products of cariogenic and non-cariogenic streptococci." Archives of Oral Biology **13**(5): 565-570.

Duckworth, R.M., Morgan, S.N. and Burchell, C.K. (1989). "Fluoride in plaque following use of dentifrices containing sodium monofluorophosphate." Journal of Dental Research **68**(2): 130-133.

Duckworth, R.M. and Morgan, S.N. (1991). "Oral fluoride retention after use of fluoride dentifrices." Caries Research **25**(2): 123-129.

Duckworth, R.M., Jones, Y., Nicholson, J., Jacobson, A.P. and Chestnutt, I.G. (1994). "Studies on plaque fluoride after use of F-containing dentifrices." Advances in Dental Research **8**(2): 202-207.

Duckworth, R.M. (2013). Pharmacokinetics in the oral cavity: fluoride and other active ingredients. Toothpastes. van Loveren, C. **23:** 125-139.

Duggal, M.S., Toumba, K.J., Amaechi, B.T., Kowash, M.B. and Higham, S.M. (2001). "Enamel demineralization *in situ* with various frequencies of carbohydrate consumption with and without fluoride toothpaste." Journal of Dental Research **80**(8): 1721-1724.

Duschner, H., Gotz, H. and Ogaard, B. (1997). "Fluoride-induced precipitates on enamel surface and subsurface areas visualised by electron microscopy and confocal laser scanning microscopy." European Journal of Oral Sciences **105**(5): 466-472.

Dye, B.A., Tan, S., Smith, V., Lewis, B.G., Barker, L.K., Thornton-Evans, G., Eke, P.I., Beltran-Aguilar, E.D., Horowitz, A.M. and Li, C.-H. (2007). "Trends in oral health status: United States, 1988-1994 and 1999-2004." Vital and health statistics. Series 11, Data from the national health survey(248): 1-92.

Edgar, W.M. (1978). "Reduction in enamel dissolution by liquorice and glycyrrhizinic acid." Journal of Dental Research **57**(1): 59-64.

Edgar, W.M., Geddes, D.A.M., Jenkins, G.N., Rugggunn, A.J. and Howell, R. (1978). "Effects of calcium glycerophosphate and sodium-fluoride on induction *in vivo* of caries-like changes in human dental enamel." Archives of Oral Biology **23**(8): 655-661.

Edgar, W.M. (1982). "Duration of response and stimulus sequence in the interpretation of plaque pH data." Journal of Dental Research **61**(10): 1126-1129.

Edgar, W.M. (1983). Distribution of acetic acid in enamel during demineralisation in HEC/lactic acid system. Demineralisation and Remineralisation of the Teeth. Leach, S.A. and Edgar, W.M. Oxford, IRL**:** 145-152.

Edgar, W.M. and Geddes, D.A.M. (1986). "Plaque acidity models for cariogenicity testing - some theoretical and practical observations." Journal of Dental Research **65**: 1498-1502.

Egerton, R.F. (2005). Physical principles of electron microscopy : an introduction to TEM, SEM, and AEM, Springer.

Ehrlich, H., Koutsoukos, P.G., Demadis, K.D. and Pokrovsky, O.S. (2008). "Principles of demineralization: modern strategies for the isolation of organic frameworks: Part I. Common definitions and history." Micron **39**(8): 1062-1091.

Eisenberg, A.D. and Marquis, R.E. (1980). "Uptake of fluoride by cells of *Streptococcus mutans* in dense suspensions." Journal of Dental Research **59**(7): 1187-1191.

Eisenberg, A.D., Wegman, M.R., Oldershaw, M.D. and Curzon, M.E.J. (1985). "Effect of fluoride, lithium or strontium on acid production by pelleted human dental plaque." Caries Research **19**(5): 454-457.

Ekstrand, K., Martignon, S. and Holm-Pedersen, P. (2008). "Development and evaluation of two root caries controlling programmes for home-based frail people older than 75 years." Gerodontology **25**(2): 67-75.

Elliott, J.C. (1997). Structure, crystal chemistry and density of enamel apatites. Dental Enamel. Chadwick, D.J. and Cardew, G. **205:** 54-72.

Elton, V., Cooper, L., Higham, S.M. and Pender, N. (2009). "Validation of enamel erosion *in vitro*." Journal of Dentistry **37**(5): 336-341.

Emami, Z., Al-Khateeb, S., de Josselin de Jong, E., Sundstrom, F., Trollsas, K. and Angmar-Mansson, B. (1996). "Mineral loss in incipient caries lesions quantified with laser fluorescence and longitudinal microradiography. A methodologic study." Acta Odontologica Scandinavica **54**(1): 8-13.

Embleton, J.V., Newman, H.N. and Wilson, M. (1998). "Influence of growth mode and sucrose on susceptibility of *Streptococcus sanguis* to amine fluorides and amine fluoride-inorganic fluoride combinations." Applied and Environmental Microbiology **64**(9): 3503-3506.

Esteves-Oliveira, M., Zezell, D.M., Ana, P.A., Yekta, S.S., Lampert, F. and Eduardo, C.P. (2011). "Dentine caries inhibition through CO_2 laser (10.6 mu m) irradiation and fluoride application, *in vitro*." Archives of Oral Biology **56**(6): 533-539.

Estrela, C., Pesce, H.F., Sidney, G.B. and Figueiredo, J.A. (1994). "Apical leakage using various sealers and root canal filling techniques." Brazilian Dental Journal **5**(1): 59-63.

Estrela, C., Sydney, G.B., Bammann, L.L. and Felippe Junior, O. (1995). "Mechanism of action of calcium and hydroxyl ions of calcium hydroxide on tissue and bacteria." Brazilian Dental Journal **6**(2): 85-90.

Featherstone, J.D.B., Duncan, J.F. and Cutress, T.W. (1979). "Mechanism for dental caries based on chemical processes and diffusion phenomena during *in vitro* caries simulation on human tooth enamel." Archives of Oral Biology **24**(2): 101-112.

Featherstone, J.D.B. and Rodgers, B.E. (1981). "Effect of acetic, lactic and other organic-acids on the formation of artificial carious lesions." Caries Research **15**(5): 377-385.

Featherstone, J.D.B., O'Reilly, M.M., Shariati, M. and Brugler, S. (1986). Enhancement of remineralization *in vitro* and *in vivo*. Factors Relating to Demineralisation and Remineralisation of the Teeth. SA., L. Oxford, IRL Press, Ltd.**:** 23-34.

Featherstone, J.D.B., Glena, R., Shariati, M. and Shields, C.P. (1990). "Dependence of *in vitro* demineralization of apatite and remineralization of dental enamel on fluoride concentration." Journal of Dental Research **69**: 620-625.

Featherstone, J.D.B. and Zero, D.T. (1992). "Laboratory and human studies to elucidate the mechanism of action of fluoride-containing dentifrices." Clinical and Biological Aspects of Dentifrices: 41 - 50.

Featherstone, J.D.B. (1999). "Prevention and reversal of dental caries: role of low level fluoride." Community Dentistry And Oral Epidemiology **27**(1): 31-40.

Featherstone, J.D.B. (2000). "The science and practice of caries prevention." Journal of the American Dental Association **131**: 887 - 899.

Fejerskov, O. and Nyvad, B. (2003). Is dental caries an infectious disease? Diagnostic and treatment consequences for the practitioner. Nordic Dentistry Yearbook. Schou, L. København, Quintessence Publishing Co, Ltd**:** 141-152.

Fejerskov, O., Kidd, E.A.M., Nyvad, B. and Baelum, V. (2008). Defining the disease and its diagnosis. Dental Caries: the Disease and its Clinical Management. Fejerskov, O. and Kidd, E.A.M. Oxford, Wiley-Blackwell**:** 3-6.

Ferreira Zandoná, A.G., Isaacs, R.L., van der Veen, M.H. and Stookey, G.K. (2000). Indiana pilot clinical study of quantitative light fluorescence. Early Detection of Dental Caries II: Proceedings of the 4th Annual Indiana Conference. Stookey, G.K. Indianapolis, Indiana.

Filoche, S.K., Zhu, M. and Wu, C.D. (2004). "*In situ* biofilm formation by multi-species oral bacteria under flowing and anaerobic conditions." Journal of Dental Research **83**(10): 802-806.

Firestone, A.R. (1982). "Human interdental plaque pH data and rat caries tests - results with the same substances." Journal of Dental Research **61**(10): 1130-1136.

Flemming, H.C., Wingender, J., Geiegbe, T. and Mayer, C. (2000). Physico-chemical properties of biofilms. Biofilms Recent Advances in their Study and Control. Evans, L.V. Amsterdam, Taylor & Francis e-Library (2004)**:** 19-34.

Fletcher, M. and Floodgate, G.D. (1973). "An electron-microscopic demonstration of an acidic polysaccharide involved in the adhesion of a marine bacterium to solid surfaces." Journal of General Microbiology **74**(2): 325-334.

Fredrickson, A.G. (1977). "Behaviour of mixed cultures of microorganisms." Annual Review of Microbiology **31**: 63-87.

Freedman, M.L. and Tanzer, J.M. (1982). Use of mutants to study the glucan-associated pathophysiology of *Streptococcus mutans*. Microbiology. Schlessinger, D. Washington, American Society for Microbiology.

Frentzen, M., Winkelstrater, C., van Benthem, H. and Koort, H.J. (1996). "The effects of pulsed ultraviolet and infra-red lasers on dental enamel." European Journal of Prosthodontics and Restorative Dentistry **4**(3): 99-104.

Friedrichs, C., Rodloff, A.C., Chhatwal, G.S., Schellenberger, W. and Eschrich, K. (2007). "Rapid identification of viridans streptococci by mass spectrometric discrimination." Journal of Clinical Microbiology **45**(8): 2392-2397.

Frostell, G. (1970). "A method for evaluation of acid potentialities of foods." Acta Odontologica Scandinavica **28**(5): 599-608.

Fujii, M., Kitasako, Y., Sadr, A. and Tagami, J. (2011). "Roughness and pH changes of enamel surface induced by soft drinks *in vitro*-applications of stylus profilometry, focus variation 3D scanning microscopy and micro pH sensor." Dental Materials Journal **30**(3): 404-410.

Galland, P. and Senger, H. (1988). "The role of flavins as photoreceptors." Journal of Photochemistry and Photobiology B - Biology **1**(3): 277-294.

Gameiro, G.H., Nouer, D.F., Cenci, M.S. and Cury, J.A. (2009). "Enamel demineralization with two forms of archwire ligation investigated using an *in situ* caries model-a pilot study." European Journal of Orthodontics **31**(5): 542-546.

Garg, A.K., Agrawal, N., Tewari, R.K., Kumar, A. and Chandra, A. (2014). "Antibiotic prescription pattern among Indian oral healthcare providers: a cross-sectional survey." Journal of Antimicrobial Chemotherapy **69**(2): 526-528.

Gaugler, R.W. and Bruton, W.F. (1982). "Fluoride concentration in dental plaque of naval recruits with and without caries." Archives of Oral Biology **27**(3): 269-272.

Geddes, D.A.M. (1972). "The production of L(+) and D(−) lactic acid and volatile acids by human dental plaque and the effect of plaque buffering and acidic strength on pH." Archives of Oral Biology **17**(3): 537-545.

Geddes, D.A.M. (1973). "Acids produced by human dental plaque metabolism *in situ*." Helvetica Odontologica Acta **17**(2): 45-45.

Geddes, D.A.M. (1975). "Acids produced by human dental plaque metabolism *in situ*." Caries Research **9**(2): 98-109.

Geddes, D.A.M. and McNee, S.G. (1982). "The effect of 0.2 per cent (48 mM) NaF rinses daily on human plaque acidogenicity *in situ* (Stephan curve) and fluoride content." Archives of Oral Biology **27**(9): 765-769.

Geddes, D.A.M., Weetman, D.A. and Featherstone, J.D.B. (1984). "Preferential loss of acetic- acid from plaque fermentation in the presence of enamel." Caries Research **18**(5): 430-433.

Geddes, D.A.M. and Bowen, W.H. (1990). "Summary of session III: fluoride in saliva and dental plaque." Journal of Dental Research **69**: 637-637.

George, K.S. and Falkler, W.A. (1992). "Coaggregation studies of the *Eubacterium* species." Oral Microbiology and Immunology **7**(5): 285-290.

Gibbons, R.J. and Socransky, S.S. (1962). "Intracellular polysaccharide storage by organisms in dental plaques - its relation to dental caries and microbial ecology of the oral cavity." Archives of Oral Biology **7**(1): 73-79.

Gibbons, R.J. (1968). "Role of extracellular bacterial polysaccharides in the caries process." Journal of Dental Research **47**(6): 926-927.

Gibbons, R.J. and Nygaard, M. (1968). "Synthesis of insoluble dextran and its significance in the formation of gelatinous deposits by plaque-forming streptococci." Archives of Oral Biology **13**(10): 1249-1262.

Gibbons, R.J. and Houte, J.V. (1975). "Bacterial adherence in oral microbial ecology." Annual Reviews of Microbiology **29**: 19-44.

Gibbons, R.J. and Hay, D.I. (1988). "Human salivary acidic proline-rich proteins and statherin promote the attachment of *Actinomyces viscosus* LY7 to apatitic surfaces." Infection and Immunity **56**(2): 439-445.

Gibbons, R.J., Hay, D.I., Cisar, J.O. and Clark, W.B. (1988). "Adsorbed salivary proline-rich protein 1 and statherin: receptors for type 1 fimbriae of *Actinomyces viscosus* T14V-J1 on apatitic surfaces." Infection and Immunity **56**(11): 2990-2993.

Gibbons, R.J. (1989). "Bacterial adhesion to oral tissues - a model for infectious diseases." Journal of Dental Research **68**(5): 750-760.

Gibbons, R.J., Hay, D.I. and Schlesinger, D.H. (1991). "Delineation of a segment of adsorbed salivary acidic proline-rich proteins which promotes adhesion of *Streptococcus gordonii* to apatitic surfaces." Infection and Immunity **59**(9): 2948-2954.

Gilbert, P., Allison, D.G., Evans, D.J., Handley, P.S. and Brown, M.R. (1989). "Growth rate control of adherent bacterial populations." Applied and Environmental Microbiology **55**(5): 1308-1311.

Gilbert, P., Das, J. and Foley, I. (1997). "Biofilm susceptibility to antimicrobials." Advances in Dental Research **11**(1): 160-167.

Gilmour, M.N. and Poole, A.E. (1967). "The fermentative capabilities of dental plaque." Caries research **1**(3): 246-260.

Glansdorff, P. and Prigogine, I. (1971). Thermodynamic Theory of Structure Stability and Fluctuations, Wiley-Blackwell.

Gmur, R., Giertsen, E., van der Veen, M.H., de Josselin de Jong, E., ten Cate, J.M. and Guggenheim, B. (2006). "*In vitro* quantitative light-induced fluorescence to measure changes in enamel mineralization." Clinical Oral Investigations **10**(3): 187-195.

Goldstein, J. (2003). Scanning electron microscopy and x-ray microanalysis., Kluwer Adacemic/Plenum Publishers.

Gotowka, T.D. (1985). "Economic growth of the dental profession: comparisons with other health care sectors." Journal of the American Dental Association **110**(2): 179-187.

Gracia, L.H., Brown, A., Rees, G.D. and Fowler, C.E. (2010). "Studies on a novel combination polymer system: *in vitro* erosion prevention and promotion of fluoride uptake in human enamel." Journal of Dentistry **38, Supplement 3**: S4-S11.

Graf, H. and Muhlemann, H.R. (1966). "Telemetry of plaque pH from interdental area." Helvetica Odontologica Acta **10**(2): 94-101.

Grando, L.J., Tames, D.R., Cardoso, A.C. and Gabilan, N.H. (1996). "*In vitro* study of enamel erosion caused by soft drinks and lemon juice in deciduous teeth analysed by stereomicroscopy and scanning electron microscopy." Caries Research **30**(5): 373-378.

Green, G.E. and Dodd, M.C. (1957). "Resistance of oral lactobacilli to sodium fluoride." Journal of the American Dental Association **54**(5): 654-656.

Groeneveld, A. and Arends, J. (1975). "Influence of pH and demineralization time on mineral content, thickness of surface layer and depth of artifical caries lesions." Caries Research **9**(1): 36-44.

Gron, P. and Brudevold, F. (1967). "The effectiveness of NaF dentifrices." Journal of Dentistry for Children **34**(2): 123-127.

Grossi, S.G. and Genco, R.J. (1998). "Periodontal disease and diabetes mellitus: a two-way relationship." Annals of Periodontology **3**(1): 51-61.

Guggenheim, B., Guggenheim, M., Gmur, R., Giertsen, E. and Thurnheer, T. (2004). "Application of the Zurich biofilm model to problems of cariology." Caries Research **38**(3): 212-222.

Gustafsson, B.E., Quensel, C.E., Lanke, L.S., Lundqvist, C., Grahnen, H., Bonow, B.E. and Krasse, B. (1954). "The Vipeholm dental caries study; the effect of different levels of carbohydrate intake on caries activity in 436 individuals observed for five years." Acta odontologica Scandinavica **11**(3-4): 232-264.

Gutow, J.H. (2005). "Halide (Cl-) quenching of quinine sulfate fluorescence: A time-resolved fluorescence experiment for physical chemistry." Journal of Chemical Education **82**(2): 302-305.

Guyton, A.C., Hall, J.E. and Schmitt, W. (1997). Secretary functions of the alimentary tract. Human physiology and mechanisms of disease. Philadelphia, Saunders **6th:** 524-536.

Habelitz, S., Marshall, S.J., Marshall Jr, G.W. and Balooch, M. (2001). "Mechanical properties of human dental enamel on the nanometre scale." Archives of Oral Biology **46**(2): 173-183.

Hafstrom-Bjorkman, U., Sundstrom, F., de Josselin de Jong, E., Oliveby, A. and Angmar-Mansson, B. (1992). "Comparison of laser fluorescence and longitudinal microradiography for quantitative assessment of *in vitro* enamel caries." Caries Research **26**(4): 241-247.

Haikel, Y., Frank, R.M. and Voegel, J.C. (1983). "Scanning electron microscopy of the human enamel surface layer of incipient carious lesions." Caries Research **17**(1): 1-13.

Hall, A.F., DeSchepper, E., Ando, M. and Stookey, G.K. (1997a). "*In vitro* studies of laser fluorescence for detection and quantification of mineral loss from dental caries." Advances in Dental Research **11**(4): 507-514.

Hall, A.F., Sadler, J.P., Strang, R., de Josselin de Jong, E., Foye, R.H. and Creanor, S.L. (1997b). "Application of transverse microradiography for measurement of mineral loss by acid erosion." Advances in Dental Research **11**(4): 420-425.

Hamada, S. and Slade, H.D. (1980). "Biology, immunology, and cariogenicity of *Streptococcus mutans*." Microbiological Reviews **44**(2): 331-384.

Hamilton, I.R. (1969). "Studies with fluoride-sensitive and fluoride-resistant strains of streptococcus salivarius. I. Inhibition of both intracellular polyglucose synthesis and degradation by fluoride." Canadian Journal of Microbiology **15**(9): 1013-1019.

Hamilton, I.R. (1976). Intracellular polysaccharide synthesis by cariogenic microorganisms. Microbial Aspects of Dental Caries: Proceedings of a Workshop on Microbial Aspects of Dental Caries: June 21- 24, 1976, St. Simons Island, Georgia. Stiles, H.M., O'Brien, T.C. and Loesche, W.J. Washington, D. C., Information Retrieval Inc.

Hamilton, I.R. (1977). "Effects of fluoride on enzymatic regulation of bacterial carbohydrate-metabolism." Caries Research **11**: 262-278.

Hamilton, I.R., Boyar, R.M. and Bowden, G.H. (1985). "Influence of pH and fluoride on properties of an oral strain of *Lactobacillus casei* grown in continuous culture." Infection and Immunity **48**(3): 664-670.

Hamilton, I.R. (1987). Effect of changing environment on sugar transport and metabolism by oral bacteria. Sugar Transport and Metabolism by Gram-positive Bacteria. Reizer, J. and Peterkofsky, A. Chichester, England, Ellis Horwood**:** 94-133.

Hamilton, I.R. and Bowden, G.H. (1988). Effect of fluoride on oral microorganisms. Fluoride in Dentistry. Ekstrand, J., Fejerskov, O. and Silverstone, L.M. Copenhagen, Munksgaard**:** 77-103.

Hamilton, I.R. (1990). "Biochemical effects of fluoride on oral bacteria." Journal of Dental Research **69**: 660-667.

Hamilton, I.R. and Bowden, G.H.W. (1996). Fluoride effects on oral bacteria. Fluoride in Dentistry, 2nd edition. Fejerskov, O., Ekstrand, J. and Burt, B.A.**:** 230-251.

Hanada, N. and Kuramitsu, H.K. (1988). "Isolation and characterization of the *Streptococcus mutans* gtfC gene, coding for synthesis of both soluble and insoluble glucans." Infection and Immunity **56**(8): 1999-2005.

Hanada, N. and Kuramitsu, H.K. (1989). "Isolation and characterization of the *Streptococcus mutans* gtfD gene, coding for primer-dependent soluble glucan synthesis." Infection and Immunity **57**(7): 2079-2085.

Handley, P.S., Hesketh, L.M. and Moumena, R.A. (1991). "Charged and hydrophobic groups are localised in the short and long tuft fibrils on *Streptococcus sanguis* strains." Biofouling **4**(1-3): 105-111.

Hara, A.T., de Magalhaes, C.S., Serra, M.C. and Rodrigues, A.L. (2002). "Cariostatic effect of fluoride-containing restorative systems associated with dentifrices on root dentin." Journal of Dentistry **30**(5-6): 205-212.

Harms, R.L., Martinez, D.R. and Griego, V.M. (1986). "Isolation and characterization of coproporphyrin produced by four subspecies of *Bacillus thuringiensis*." Applied and Environmental Microbiology **51**(3): 481-486.

Harold, F.M. (1977). "Membranes and energy transduction in bacteria." Current Opinions in Bioenergetics: 83-149.

Harris, G.S., Michalek, S.M. and Curtiss, R. (1992). "Cloning of a locus involved in *Streptococcus mutans* intracellular polysaccharide accumulation and virulence testing of an intracellular polysaccharide-deficient mutant." Infection and Immunity **60**(8): 3175-3185.

Hata, S. and Mayanagi, H. (2003). "Acid diffusion through extracellular polysaccharides produced by various mutants of *Streptococcus mutans*." Archives of Oral Biology **48**(6): 431-438.

Havarstein, L.S., Gaustad, P., Nes, I.F. and Morrison, D.A. (1996). "Identification of the streptococcal competence-pheromone receptor." Molecular Microbiology **21**(4): 863-869.

Hay, D.I., Moreno, E.C. and Schlesinger, D.H. (1979). "Phosphoprotein inhibitors of calcium phosphate precipitation from salivary secretions." Inorganic Perspectives in Biology and Medicine **2**(4): 271-285.

Hayes, M.L. and Hyatt, A.T. (1974). "The decarboxylation of amino acids by bacteria derived from human dental plaque." Archives of Oral Biology **19**(5): 361-369.

Head, J. (1912). "A study of saliva and its action on tooth enamel in reference to its hardening and softening." Journal of the American Medical Association **59**: 2118-2122.

Hefti, A. and Schmid, R. (1979). "Effect on caries incidence in rats of increasing dietary sucrose levels." Caries Research **13**(5): 298-300.

Heinrich-Weltzien, R., Kuhnisch, J., van der Veen, M., de Jong, E.D. and Stosser, L. (2003). "Quantitative light-induced fluorescence (QLF) - A potential method for the dental practitioner." Quintessence International **34**(3): 181-188.

Hemingway, C.A., White, A.J., Shellis, R.P., Addy, M., Parker, D.M. and Barbour, M.E. (2010). "Enamel erosion in dietary acids: inhibition by food proteins *in vitro*." Caries Research **44**(6): 525-530.

Hengtrakool, C., Pearson, G.J. and Wilson, M. (2006). "Interaction between GIC and *S. sanguis* biofilms: Antibacterial properties and changes of surface hardness." Journal of Dentistry **34**(8): 588-597.

Henrici, A.T. (1933). "Studies of freshwater bacteria I A direct microscopic technique." Journal of Bacteriology **25**(3): 277-287.

Henrissat, B. (1998). "Glycosidase families." Biochemical Society Transactions **26**(2): 153-156.

Herles, S., Olsen, S., Afflitto, J. and Gaffar, A. (1994). "Chemostat flow cell system: an *in vitro* model for the evaluation of antiplaque agents." Journal of Dental Research **73**(11): 1748-1755.

Heurich, E., Beyer, M., Jandt, K.D., Reichert, J., Herold, V., Schnabelrauch, M. and Sigusch, B.W. (2010). "Quantification of dental erosion—A comparison of stylus profilometry and confocal laser scanning microscopy (CLSM)." Dental Materials **26**(4): 326-336.

Hill, K.E., Malic, S., McKee, R., Rennison, T., Harding, K.G., Williams, D.W. and Thomas, D.W. (2010). "An *in vitro* model of chronic wound biofilms to test wound dressings and assess antimicrobial susceptibilities." Journal of Antimicrobial Chemotherapy **65**(6): 1195-1206.

Hoffman, S., McEwan, W.S. and Drew, C.M. (1969). "Scanning electron microscope studies of dental enamel." Journal of Dental Research **48**(2): 242-250.

Høiby, N., Frederiksen, B. and Pressler, T. (2005). "Eradication of early *Pseudomonas aeruginosa* infection." Journal of Cystic Fibrosis **4**: 49-54.

Holmen, L., Thylstrup, A., Ogaard, B. and Kragh, F. (1985). "A scanning electron microscopic study of progressive stages of enamel caries *in vivo*." Caries Research **19**(4): 355-367.

Holmen, L., Thylstrup, A. and Artun, J. (1987). "Surface changes during the arrest of active enamel carious lesions *in vivo* - a scanning electron microscope study." Acta Odontologica Scandinavica **45**(6): 383-390.

Honkala, E. and Tala, H. (1987). "Total sugar consumption and dental caries in Europe--an overview." International Dental Journal **37**(3): 185-191.

Hoogenkamp, M.A., van Loveren, C. and ten Cate, J.M. (2003). "The effects of sodium fluoride on a nine-species consortium grown in constant-depth film fermenter (CDFF) biofilm." Journal of Dental Research **82**: B284-B284.

Hope, C.K., Clements, D. and Wilson, M. (2002). "Determining the spatial distribution of viable and nonviable bacteria in hydrated microcosm dental plaques by viability profiling." Journal of Applied Microbiology **93**(3): 448-455.

Hope, C.K. and Wilson, M. (2003). "Measuring the thickness of an outer layer of viable bacteria in an oral biofilm by viability mapping." Journal of Microbiological Methods **54**(3): 403-410.

Hope, C.K., Petrie, A. and Wilson, M. (2005). "Efficacy of removal of sucrose-supplemented interproximal plaque by electric toothbrushes in an *in vitro* model." Applied and Environmental Microbiology **71**(2): 1114-1116.

Hope, C.K. and Wilson, M. (2006). "Biofilm structure and cell vitality in a laboratory model of subgingival plaque." Journal of Microbiological Methods **66**(3): 390-398.

Hope, C.K., de Jong, E.D., Field, M.R.T., Valappil, S.P. and Higham, S.M. (2011). "Photobleaching of red fluorescence in oral biofilms." Journal of Periodontal Research **46**(2): 228-234.

Hope, C.K., Bakht, K., Burnside, G., Martin, G.C., Burnett, G., de Jong, E.D. and Higham, S.M. (2012). "Reducing the variability between constant-depth film fermenter experiments when modelling oral biofilm." Journal of Applied Microbiology **113**(3): 601-608.

Hoshino, E., Yamada, T. and Araya, S. (1976). "Lactate degradation by a strain of *Neisseria* isolated from human dental plaque." Archives of Oral Biology **21**(11): 677-683.

Hove, L., Holme, B., Ogaard, B., Willumsen, T. and Tveit, A.B. (2006). "The protective effect of TiF_4,SnF_2 and NaF on erosion of enamel by hydrochloric acid *in vitro* measured by white light interferometry." Caries Research **40**(5): 440-443.

Hudson, D.E., Donoghue, H.D. and Perrons, C.J. (1986). "A laboratory microcosm (artificial mouth) for the culture and continuous pH measurement of oral bacteria on surfaces." Journal of Applied Bacteriology **60**(4): 301-310.

Humphrey, B.A., Dickson, M.R. and Marshall, K.C. (1979). "Physicochemical and *in situ* observations on the adhesion of gliding bacteria to surfaces." Archives of Microbiology **120**(3): 231-238.

Humphreys, G.J. and McBain, A.J. (2013). "Continuous culture of sessile human oropharyngeal microbiotas." Journal of Medical Microbiology **62**: 906-916.

Igarashi, K., Lee, I.K. and Schachtele, C.F. (1989). "Comparison of *in vivo* human dental plaque pH changes with artificial fissures and at interproximal sites." Caries Research **23**(6): 417-422.

Igarashi, K., Lee, I.K. and Schachtele, C.F. (1990). "Effect of dental plaque age and bacterial composition on the pH of artificial fissures in human volunteers." Caries Research **24**(1): 52-58.

Imfeld, T. (1977). "Evaluation of the cariogenicity of confectionery by intra-oral wire-telemetry." Schweizerische Monatsschrift fur Zahnheilkunde **87**(5): 437-464.

Imfeld, T. (1983). "Scanning electron microscopy of plaque colonization on indwelling glass electrodes." Caries Research **17**(5): 461-465.

James, G.A., Beaudette, L. and Costerton, J.W. (1995). "Interspecies bacterial interactions in biofilms." Journal of Industrial Microbiology **15**(4): 257-262.

Jenkins, G.N. (1959). "The effect of pH on the fluoride inhibition of salivary acid production." Archives of Oral Biology **1**(1): 33-41.

Jenkins, G.N. (1961). "A critique of the proteolysis-chelation theory of caries." British Dental Journal **111**: 311-330.

Jenkins, G.N. and Edgar, W.M. (1969). "Distribution and metabolic effects of human plaque fluorine." Archives of Oral Biology **14**(1): 105-119.

Jenkins, G.N. and Edgar, W.M. (1977). "Distribution and forms of F in saliva and plaque." Caries Research **11**: 226-237.

Jensen, M.E. and Schachtele, C.F. (1983). "The acidogenic potential of reference foods and snacks at interproximal sites in the human dentition." Journal of Dental Research **62**(8): 889-892.

Johansen, E. (1965). "Electron microscopic and chemical studies of carious lesions with reference to organic phase of affected tissues." Annals of the New York Academy of Sciences **131**(A2): 776-785.

Johnson, M.C., Bozzola, J.J., Shechmeister, I.L. and Shklair, I.L. (1977). "Biochemical study of the relationship of extracellular glucan to adherence and cariogenicity in *Streptococcus mutans* and an extracellular polysaccharide mutant." Journal of Bacteriology **129**(1): 351-357.

Johnsson, A., Kjeldstad, B. and Melø, T.B. (1987). "Fluorescence from pilosebaceous follicles." Archives of Dermatological Research **279**(3): 190-193.

Jones, H.C., Roth, I.L. and Sanders, W.M. (1969). "Electron microscopic study of a slime layer." Journal of Bacteriology **99**(1): 316-325.

Jordan, H.V. (1965). "Bacteriological aspects of experimental dental caries." Annals of the New York Academy of Sciences **131**(2): 905-912.

Josey, A.L., Meyers, I.A., Romaniuk, K. and Symons, A.L. (1996). "The effect of a vital bleaching technique on enamel surface morphology and the bonding of composite resin to enamel." Journal of Oral Rehabilitation **23**(4): 244-250.

Joyston-Bechal, S. and Kidd, E.A.M. (1982). "Effect on fluoride uptake by enamel and on the progress of artificially produced caries-like lesions of applying successively 2 different fluoride solutions." Caries Research **16**(1): 34-41.

Julihn, A., Agholme, M.B., Grindefjord, M. and Modeer, T. (2006). "Risk factors and risk indicators associated with high caries experience in Swedish 19-years-olds." Acta Odontologica Scandinavica **64**(5): 267-273.

Kanapka, J.A. and Hamilton, I.R. (1971). "Fluoride inhibition of enolase activity *in vivo* and its relationship to the inhibition of glucose-6-P formation in *Streptococcus salivarius*." Archives of Biochemistry and Biophysics **146**(1): 167-174.

Kashket, S. and Rodriguez, V.M. (1976). "Fluoride accumulation by a strain of human oral *Streptococcus sanguis*." Archives of Oral Biology **21**(8): 459-464.

Kashket, S., Rodriguez, V.M. and Bunick, F.J. (1977). "Inhibition of glucose-utilisation in oral streptococci by low concentrations of fluoride." Caries Research **11**(6): 301-307.

Kashket, S. and Bunick, F.J. (1978). "Binding of fluoride in oral streptococci." Archives of Oral Biology **23**(11): 993-996.

Kashket, S. and Kashket, E.R. (1985). "Dissipation of the proton motive force in oral streptococci by fluoride." Infection and Immunity **48**(1): 19-22.

Kashket, S. and Preman, R.J. (1985). "Fluoride uptake and fluoride resistance in oral streptococci." Journal of Dental Research **64**(11): 1290-1292.

Kashket, S., Yaskell, T. and Murphy, J.E. (1994). "Delayed effect of wheat starch in foods on the intraoral demineralization of enamel." Caries Research **28**(4): 291-296.

Katayama, T., Edgar, W.M., Jenkins, G.N. and Johnson, B. (1981). "Preliminary fractionation of fluoride binding constituents of human oral *Streptococcus sanguis*." Archives of Oral Biology **26**(4): 341-342.

Kawashima, J., Nakajo, K., Washio, J., Mayanagi, G., Shimauchi, H. and Takahashi, N. (2013). "Fluoride-sensitivity of growth and acid production of oral *Actinomyces*: comparison with oral *Streptococcus*." Microbiology and Immunology **57**(12): 797-804.

Kazor, C.E., Mitchell, P.M., Lee, A.M., Stokes, L.N., Loesche, W.J., Dewhirst, F.E. and Paster, B.J. (2003). "Diversity of bacterial populations on the tongue dorsa of patients with halitosis and healthy patients." Journal of Clinical Microbiology **41**(2): 558-563.

Keene, H. (1980). History of dental caries in human populations: the first million years. Symposium and Workshop on Animal Models in Cariology, Sturbridge, Massachusets.

Keevil, C.W., Bradshaw, D.J., Dowsett, A.B. and Feary, T.W. (1987). "Microbial film formation: dental plaque deposition on acrylic tiles using continuous culture techniques." Journal of Applied Bacteriology **62**(2): 129-138.

Kidd, E.A.M. and Joyston-Bechal, S. (1982). "Relationship between the extent of the initial lesion and the inhibitory effect of APF on the progression of caries-like lesions *in vitro*." Caries Research **16**(1): 42-46.

Kilian, M., Thylstrup, A. and Fejerskov, O. (1979). "Predominant plaque flora of Tanzanian children exposed to high and low water fluoride concentrations." Caries Research **13**(6): 330-343.

Kim, M.J., Lee, S.H., Lee, N.Y. and Lee, I.H. (2013). "Evaluation of the effect of PVA tape supplemented with 2.26% fluoride on enamel demineralization using microhardness assessment and scanning electron microscopy: In vitro study." Archives of Oral Biology **58**(2): 160-166.

Kim, Y.S., Lee, E.S., Kwon, H.K. and Kim, B.I. (2014). "Monitoring the maturation process of a dental microcosm biofilm using the Quantitative Light-induced Fluorescence-Digital (QLF-D)." Journal of Dentistry **42**(6): 691-696.

Kinniment, S.L., Wimpenny, J.W., Adams, D. and Marsh, P.D. (1996a). "The effect of chlorhexidine on defined, mixed culture oral biofilms grown in a novel model system." Journal of Applied Bacteriology **81**(2): 120-125.

Kinniment, S.L., Wimpenny, J.W., Adams, D. and Marsh, P.D. (1996b). "Development of a steady-state oral microbial biofilm community using the constant-depth film fermenter." Microbiology **142 Pt 3**: 631-638.

Kirkham, J., Robinson, C., Strong, M. and Shore, R.C. (1994). "Effects of frequency and duration of acid exposure on demineralisation/remineralisation behaviour of human enamel *in vitro*." Caries Research **28**(1): 9-13.

Kjeldstad, B., Johnsson, A. and Sandberg, S. (1984). "Influence of pH on porphyrin production in *Propionibacterium acnes*." Archives of Dermatological Research **276**(6): 396-400.

Kleber, C.J. and Putt, M.S. (1995). "Investigation of the effects of aluminum mouthrinses on rat dental caries and plaque." Caries Research **29**(3): 237-242.

Kleinberg, I. (1967). "Effect of urea concentration on human plaque pH levels *in situ*." Archives of Oral Biology **12**(12): 1475-1484.

Kleinberg, I., Jenkins, G.N., Chatterjee, R. and Wijeyeweera, L. (1982). "The antimony pH electrode and its role in the assessment and interpretation of dental plaque pH." Journal of Dental Research **61**(10): 1139-1147.

Kleinberg, I. (2002). "A mixed-bacteria ecological approach to understanding the role of oral bacteria in dental caries causation: an alternative to *Streptococcus mutans* and the specific plaque hypothesis." Critical Reviews in Oral Biology & Medicine **13**(2): 108-125.

Ko, C.C., Tantbirojn, D., Wang, T. and Douglas, W.H. (2000). "Optical scattering power for characterization of mineral loss." Journal of Dental Research **79**(8): 1584-1589.

Kobayashi, H. (1987). Regulation of cytoplasmic pH in streptococci. Sugar Transport and Metabolism by Gram-positive Bacteria. Reizer, J. and Peterkofsky, A. Chichester , England, Ellis Horwood**:** 255-269.

Kodukula, P.S., Prakasam, T.B.S. and Antonisen, A.C. (1988). Role of pH in biological wastewater treatment process. Physiological Models in Microbiology. 1st edition. Bazin, M.J. and Prosser, J.I. Boca Raton, CRC Press**:** 114-134.

Kolenbrander, P.E., Andersen, R.N. and Moore, L.V.H. (1989). "Coaggregation of *Fusobacterium nucleatum*, *Selenomonas flueggei*, *Selenomonas infelix*, *Selenomonas noxia*, and *Selenomonas sputigena* with strains from 11 genera of oral bacteria." Infection and Immunity **57**(10): 3194-3203.

Kolenbrander, P.E. and London, J. (1992). "Ecological significance of coaggregation among oral bacteria." Advances in Microbial Ecology **12**: 183-217.

Kolenbrander, P.E. and London, J. (1993). "Adhere today, here tomorrow - oral bacterial adherence." Journal of Bacteriology **175**(11): 3247-3252.

Kolenbrander, P.E. (2000). "Oral microbial communities: biofilms, interactions, and genetic systems." Annual Review of Microbiology **54**(1): 413-437.

Konig, K., Hibst, R., Meyer, H., Flemming, G. and Schneckenburger, H. (1993). "Laser-induced autofluorescence of carious regions of human teeth and caries-involved bacteria." Proceedings of Dental Applications of Lasers **2080**: 170-180.

Konig, K., Schneckenburger, H., Hemmer, J., Tromberg, B. and Steiner, R. (1994). *In vivo* fluorescence detection and imaging of porphyrin-producing bacteria in the human skin and in the oral cavity for diagnosis of acne-vulgaris, caries and squamous-cell carcinoma. Advances in Laser and Light Spectroscopy to Diagnose Cancer and Other Diseases. Alfano, R.R. and Katzir, A. **2135:** 129-138.

Konig, K.G., Schmid, P. and Schmid, R. (1968). "An apparatus for frequency-controlled feeding of small rodents and its use in dental caries experiments." Archives of Oral Biology **13**(1): 13-26.

Koo, H., Hayacibara, M.F., Schobel, B.D., Cury, J.A., Rosalen, P.L., Park, Y.K., Vacca-Smith, A.M. and Bowen, W.H. (2003). "Inhibition of *Streptococcus mutans* biofilm accumulation and polysaccharide production by apigenin and tt-farnesol." Journal of Antimicrobial Chemotherapy **52**(5): 782-789.

Koo, H., Schobel, B., Scott-Anne, K., Watson, G., Bowen, W.H., Cury, J.A., Rosalen, P.L. and Park, Y.K. (2005). "Apigenin and tt-farnesol with fluoride effects on *S. mutans* biofilms and dental caries." Journal of Dental Research **84**(11): 1016-1020.

Koo, H. (2008). "Strategies to enhance the biological effects of fluoride on dental biofilms." Advances in Dental Research **20**(1): 17-21.

Koulourides, T. (1977). "Effect of 1 ppm fluoride on microradiographic profile of experimental enamel lesions." Journal of Dental Research **56**: A110-A110.

Koulourides, T. and Cameron, B. (1980). "Enamel remineralisation as a factor in the pathogenesis of dental caries." Journal of Oral Pathology & Medicine **9**(5): 255-269.

Kreth, J., Merritt, J., Shi, W.Y. and Qi, F.X. (2005). "Competition and coexistence between *Streptococcus mutans* and *Streptococcus sanguinis* in the dental biofilm." Journal of Bacteriology **187**(21): 7193-7203.

Küchenmeister, F. (1857). On animal and vegetable parasites of the human body, a manual of their natural history, diagnosis, and treatment. London, Printed for the Sydenham Society.

Kuramitsu, H.K. (1975). "Characterization of extracellular glucosyltransferase activity of *Steptococcus mutans*." Infection and Immunity **12**(4): 738-749.

Lagerlof, F., Dawes, R. and Dawes, C. (1984). "Salivary clearance of sugar and its effects on pH changes by *Streptococcus mitior* in an artificial mouth." Journal of Dental Research **63**(11): 1266-1270.

Lagerweij, M.D., Damen, J.J.M. and ten Cate, J.M. (1996). "Demineralization of dentine grooves *in vitro*." Caries Research **30**(3): 231-236.

Lagerweij, M.D., van der Veen, M.H., Ando, M., Lukantsova, L. and Stookey, G.K. (1999). "The validity and repeatability of three light-induced fluorescence systems: an *in vitro* study." Caries Research **33**(3): 220-226.

Lagerweij, M.D. and ten Cate, J.M. (2006). "Acid susceptibility at various depths of pH-cycled enamel and dentine specimens." Caries Research **40**(1): 33-37.

Lakowicz, J.R. and Weber, G. (1973). "Quenching of fluorescence by oxygen - probe for structural fluctuations in macromolecules." Biochemistry **12**(21): 4161-4170.

Lamb, J.F., Ingram, C.G., Johnston, I.A. and Pitman, R.M. (1991). Gastrointestinal system and nutrition. Essentials of physiology. Oxford, Blackwell Scientific Publications. **3rd:** 91-115.

Lambrou, D., Larsen, M.J., Fejerskov, O. and Tachos, B. (1981). "The effect of fluoride in saliva on remineralisation of dental enamel in humans." Caries Research **15**(5): 341-345.

Lammers, P.C., Borggreven, J., Driessens, F.C.M. and Vanthof, M.A. (1992). "Acid-susceptibility of lesions in bovine enamel after remineralization in the presence of fluoride and or carbonate." Caries Research **26**(1): 1-7.

Larrimore, S., Murchison, H., Shiota, T., Michalek, S.M. and Curtiss, R. (1983). "*In vitro* and *in vivo* complementation of *Streptococcus mutans* mutants defective in adherence." Infection and Immunity **42**(2): 558-566.

Larsen, M.J. (1974). "Chemically induced *in vitro* lesions in dental enamel." Scandinavian Journal of Dental Research **82**(7): 496-509.

Larsen, M.J. and Richards, A. (2001). "The influence of saliva on the formation of calcium fluoride-like material on human dental enamel." Caries Research **35**(1): 57-60.

Laue, H., Schenk, A., Li, H., Lambertsen, L., Neu, T.R., Molin, S. and Ullrich, M.S. (2006). "Contribution of alginate and levan production to biofilm formation by *Pseudomonas syringae*." Microbiology **152**(10): 2909-2918.

Lawrence, J.R., Korber, D.R., Hoyle, B.D., Costerton, J.W. and Caldwell, D.E. (1991). "Optical sectioning of microbial biofilms." Journal of Bacteriology **173**(20): 6558-6567.

Leber, T. and Rottenstein, J.B. (1867). Untersuchungen über die Caries der Zähne von Th. Leber und J.B. Rottenstein : Mit 2 lithogr. Tafeln. Berlin, Hirschwald.

Ledder, R.G., Gilbert, P., Pluen, A., Sreenivasan, P.K., De Vizio, W. and McBain, A.J. (2006). "Individual microflora beget unique oral microcosms." Journal of Applied Microbiology **100**(5): 1123-1131.

Lee, E.S., Kang, S.M., Ko, H.Y., Kwon, H.K. and Kim, B.I. (2013). "Association between the cariogenicity of a dental microcosm biofilm and its red fluorescence detected by Quantitative Light-induced Fluorescence-Digital (QLF-D)." Journal of Dentistry **41**(12): 1264-1270.

Lehninger, A.L., Nelson, D.L. and Cox, M.M. (2013). Lehninger principles of biochemistry, New York: W.H. Freeman

Leitao, T.J., Andalo Tenuta, L.M., Ishi, G. and Cury, J.A. (2012). "Calcium binding to *S. mutans* grown in the presence or absence of sucrose." Brazilian Oral Research **26**(2): 100-105.

Lemos, J.A. and Burne, R.A. (2008). "A model of efficiency: stress tolerance by *Streptococcus mutans*." Microbiology **154**(11): 3247-3255.

Lennon, A.M., Buchalla, W., Switalski, L. and Stookey, G.K. (2002). "Residual caries detection using visible fluorescence." Caries Research **36**(5): 315-319.

Lennon, A.M., Buchalla, W., Brune, L., Zimmermann, O., Gross, U. and Attin, T. (2006). "The ability of selected oral microorganisms to emit red fluorescence." Caries Research **40**(1): 2-5.

Leung, D., Spratt, D.A., Pratten, J., Gulabivala, K., Mordan, N.J. and Young, A.M. (2005). "Chlorhexidine-releasing methacrylate dental composite materials." Biomaterials **26**(34): 7145-7153.

Leverett, D.H., Featherstone, J.D.B., Proskin, H.M., Adair, S.M., Eisenberg, A.D., Mundorff-Shrestha, S.A., Shields, C.P., Shaffer, C.L. and Billings, R.J. (1993a). "Caries risk assessment by a cross-sectional discrimination model." Journal of Dental Research **72**(2): 529-537.

Leverett, D.H., Proskin, H.M., Featherstone, J.D.B., Adair, S.M., Eisenberg, A.D., Mundorff-Shrestha, S.A., Shields, C.P., Shaffer, C.L. and Billings, R.J. (1993b). "Caries risk assessment in a longitudinal discrimination study." Journal of Dental Research **72**(2): 538-543.

Levine, M.J., Tabak, L.A., Reddy, M.S. and Mandel, I.D. (1985). "Nature of salivary pellicles in microbial adherence: role of salivary mucins." Molecular Basis of Oral Microbial Adhesion: 125-130.

Lewis, K. (2001). "Riddle of biofilm resistance." Antimicrobial Agents and Chemotherapy **45**(4): 999-1007.

Li, J., Helmerhorst, E.J., Leone, C.W., Troxler, R.F., Yaskell, T., Haffajee, A.D., Socransky, S.S. and Oppenheim, F.G. (2004). "Identification of early microbial colonizers in human dental biofilm." Journal of Applied Microbiology **97**(6): 1311-1318.

Li, Y. and Burne, R.A. (2001). "Regulation of the *gtfBC* and *ftf* genes of *Streptococcus mutans* in biofilms in response to pH and carbohydrate." Microbiology **147**(10): 2841-2848.

Li, Y.H. and Bowden, G.H. (1994). "Characteristics of accumulation of oral gram-positive bacteria on mucin conditioned glass surfaces in a model system." Oral Microbiology & Immunology **9**(1): 1-11.

Li, Y.H., Chen, Y.Y.M. and Burne, R.A. (2000). "Regulation of urease gene expression by *Streptococcus salivarius* growing in biofilms." Environmental Microbiology **2**(2): 169-177.

Li, Y.H., Tang, N., Aspiras, M.B., Lau, P.C.Y., Lee, J.H., Ellen, R.P. and Cvitkovitch, D.G. (2002). "A quorum-sensing signaling system essential for genetic competence in *Streptococcus mutans* is involved in biofilm formation." Journal of Bacteriology **184**(10): 2699-2708.

Lima, T.J., Ribeiro, C.C.C., Tenuta, L.M.A. and Cury, J.A. (2008). "Low-fluoride dentifrice and caries lesion control in children with different caries experience: a randomized clinical trial." Caries Research **42**(1): 46-50.

Lindfors, B. and Lagerlof, F. (1988). "Effect of sucrose concentration in saliva after a sucrose rinse on the hydronium ion concentration in dental plaque." Caries Research **22**(1): 7-10.

Lingstrom, P., Imfeld, T. and Birkhed, D. (1993). "Comparison of three different methods for measurement of plaque-pH in humans after consumption of soft bread and potato chips." Journal of Dental Research **72**(5): 865-870.

Lingstrom, P., Birkhed, D., Ruben, J. and Arends, J. (1994). "Effect of frequent consumption of starchy food items on enamel and dentin demineralization and on plaque pH *in situ*." Journal of Dental Research **73**(3): 652-660.

Lippert, F., Butler, A., Lynch, R.J.M. and Hara, A.T. (2012). "Effect of fluoride, lesion baseline severity and mineral distribution on lesion progression." Caries Research **46**(1): 23-30.

Lippert, F. (2013). "An introduction to toothpaste - its purpose, history and ingredients." Toothpastes **23**: 1-14.

Listgarten, M.A. (1976). "Structure of microbial flora associated with periodontal health and disease in man - light and electron- microscopic study." Journal of Periodontology **47**(1): 1-18.

Loesche, W.J. and Henry, C.A. (1967). "Intracellular microbial polysaccharide production and dental caries in a Guatemalan Indian village." Archives of Oral Biology **12**(2): 189-194.

Loesche, W.J., Murray, R.J. and Mellberg, J.R. (1973). "Effect of topical acidulated fluoride on percentage of *Streptococcus mutans* and *Streptococcus sanguis* in interproximal plaque samples." Caries Research **7**(4): 283-296.

Loesche, W.J., Syed, S.A., Murray, R.J. and Mellberg, J.R. (1975). "Effect of topical acidulated phosphate fluoride on percentage of *Streptococcus mutans* and *Streptococcus sanguis* in plaque. 2. Pooled occlusal and pooled approximal samples." Caries Research **9**(2): 139-155.

Loesche, W.J., Schumann, P.R., Edgar, W.M. and Kanapka, J. (1981). "Sampling compared to antimony microelectrode methods for plaque pH determination." Journal of Dental Research **60**: 536-536.

Loesche, W.J. (1986). "Role of *Streptococcus mutans* in human dental decay." Microbiological Reviews **50**(4): 353-380.

Loesche, W.J. (1992). "The specific plaque hypothesis and the antimicrobial treatment of periodontal disease." Dental Update **19**(2): 68, 70-62, 74.

Lustmann, J., Lewinepstein, J. and Shteyer, A. (1976). "Scanning electron microscopy of dental calculus." Calcified Tissue Research **21**(1): 47-55.

Lynch, R.J.M., Navada, R. and Walia, R. (2004). "Low-levels of fluoride in plaque and saliva and their effects on the demineralisation and remineralisation of enamel; role of fluoride toothpastes." International Dental Journal **54**(S5): 304-309.

Madhwani, T. and McBain, A.J. (2011). "Bacteriological effects of a *Lactobacillus reuteri* probiotic on *in vitro* oral biofilms." Archives of Oral Biology **56**(11): 1264-1273.

Magitot, E. (1867). Traité de la carie dentaire : recherches expérimentales et thérapeutiques sur la carie dentaire, par le Dr É. Magitot. Paris, J.-B. Baillière.

Maia, L.C., de Souza, I.P.R. and Cury, J.A. (2003). "Effect of a combination of fluoride dentifrice and varnish on enamel surface rehardening and fluoride uptake *in vitro*." European Journal of Oral Sciences **111**(1): 68-72.

Malic, S., Hill, K.E., Playle, R., Thomas, D.W. and Williams, D.W. (2011). "*In vitro* interaction of chronic wound bacteria in biofilms." Journal of Wound Care **20**(12): 569-+.

Maltz, M. and Emilson, C.G. (1982). "Susceptibility of oral bacteria to various fluoride salts." Journal of Dental Research **61**(6): 786-790.

Manly, R.S. and Harrington, D.P. (1959). "Solution rate of tooth enamel in an acetate buffer." Journal of dental research **38**: 910-919.

Manly, R.S. and Richardson, D.T. (1968). "Metabolism of levan by oral samples." Journal of Dental Research **47**(6): 1080-1086.

Margolis, H.C., Moreno, E.C. and Murphy, B.J. (1985). "Importance of high pKa acids in cariogenic potential of plaque." Journal of Dental Research **64**(5): 786-792.

Margolis, H.C. (1990). "An assessment of recent advances in the study of the chemistry and biochemistry of dental plaque fluid." Journal of Dental Research **69**(6): 1337-1342.

Margolis, H.C. and Moreno, E.C. (1992). "Composition of pooled plaque fluid from caries-free and caries-positive individuals following sucrose exposure." Journal of Dental Research **71**(11): 1776-1784.

Marinho, V.C., Higgins, J.P., Sheiham, A. and Logan, S. (2003). "Fluoride toothpastes for preventing dental caries in children and adolescents." Cochrane Database of Systematic Reviews(1): CD002278.

Marquis, R.E. (1977). "Inhibition of streptococcal adenosine triphosphatase by fluoride." Journal of Dental Research **56**(6): 704.

Marquis, R.E., Bender, G.R., Murray, D.R. and Wong, A. (1987). "Arginine deiminase system and bacterial adaptation to acid environments." Applied and Environmental Microbiology **53**(1): 198-200.

Marquis, R.E. (1990). "Diminished acid tolerance of plaque bacteria caused by fluoride." Journal of Dental Research **69**: 672-675.

Marquis, R.E. (1995). "Antimicrobial actions of fluoride for oral bacteria." Canadian Journal of Microbiology **41**(11): 955-964.

Marquis, R.E., Clock, S.A. and Mota-Meira, M. (2003). "Fluoride and organic weak acids as modulators of microbial physiology." FEMS Microbiology Reviews **26**(5): 493-510.

Marsh, P. and Bradshaw, D. (1997). "Physiological approaches to the control of oral biofilms." Advances in Dental Research **11**(1): 176-185.

Marsh, P. and Martin, M.V. (1999). Oral Microbiology, Oxford: Wright.

Marsh, P.D. (1989). "Host defenses and microbial homeostasis - role of microbial interactions." Journal of Dental Research **68**: 1567-1575.

Marsh, P.D. (1991). "Sugar, fluoride, pH and microbial homeostasis in dental plaque." Proceedings of Finnish Dental Society **87**(4): 515-525.

Marsh, P.D. (1992). "Microbiological aspects of the chemical control of plaque and gingivitis." Journal of Dental Research **71**(7): 1431-1438.

Marsh, P.D. (1994). "Microbial ecology of dental plaque and its significance in health and disease." Advances in Dental Research **8**(2): 263-271.

Marsh, P.D. (1995). "The role of continuous culture in modelling the human microflora." Journal of Chemical Technology & Biotechnology **64**(1): 1-9.

Marsh, P.D. and Bradshaw, D.J. (1995). "Dental plaque as a biofilm." Journal of Industrial Microbiology **15**(3): 169-175.

Marsh, P.D. (2000). "Role of the oral microflora in health." Microbial Ecology in Health and Disease **12**(3): 130-137.

Marsh, P.D. (2003a). "Are dental diseases examples of ecological catastrophes?" Microbiology **149**(2): 279-294.

Marsh, P.D. (2003b). "Plaque as a biofilm: pharmacological principles of drug delivery and action in the sub- and supragingival environment." Oral Diseases **9**: 16-22.

Marsh, P.D. and Nyvad, B. (2008). The oral microflora and biofilms on teeth. Dental Caries: the Disease and its Clinical Management. Fejerskov, O. and Kidd, E.A.M. Oxford, Wiley-Blackwell**:** 161-188.

Marsh, P.D., Moter, A. and Devine, D.A. (2011). "Dental plaque biofilms: communities, conflict and control." Periodontology 2000 **55**: 16-35.

Marsh, P.D. (2012). "Contemporary perspective on plaque control." British Dental Journal **212**(12): 601-606.

Marshall, G.W., Olson, L.M. and Lee, C.V. (1975). "SEM investigation of variability of enamel surfaces after simulated clinical acid etching for pit and fissure sealants." Journal of Dental Research **54**(6): 1222-1231.

Marthaler, T.M. (1990). "Changes in the prevalence of dental caries: how much can be attributed to changes in diet?" Caries Research **24 Suppl 1**: 3-15; discussion 16-25.

Matharu, S., Spratt, D.A., Pratten, J., Ng, Y.L., Mordan, N., Wilson, M. and Gulabivala, K. (2001). "A new *in vitro* model for the study of microbial microleakage around dental restorations: a preliminary qualitative evaluation." International Endodontic Journal **34**(7): 547-553.

Mattos-Graner, R.O., Smith, D.J., King, W.F. and Mayer, M.P.A. (2000). "Water-insoluble glucan synthesis by mutans streptococcal strains correlates with caries incidence in 12- to 30-month-old children." Journal of Dental Research **79**(6): 1371-1377.

McBain, A.J. (2009). "Chapter 4: *in vitro* biofilm models: an overview." Advances in Applied Microbiology **69**: 99-132.

McBride, B.C. and Gisslow, M.T. (1977). "Role of sialic-acid in saliva-induced aggregation of *Streptococcus sanguis*." Infection and Immunity **18**(1): 35-40.

McDowell, H., Gregory, T.M. and Brown, W.E. (1977). "Solubility of $Ca_5(PO_4)_3OH$ in system $Ca(OH)_2$-H_3PO_4-H_2O at 5 degrees C, 15 degrees C, 25 degrees C and 37 degrees C." Journal of Research of the National Bureau of Standards Section A - Physics and Chemistry **81**(2-3): 273-281.

McKnight-Hanes, C., Myers, D.R., Salama, F.S., Thompson, W.O. and Barenie, J.T. (1990). "Comparing treatment options for occlusal surfaces utilising an invasive index." Pediatric Dentistry **12**(4): 241-245.

McNee, S.G., Geddes, D.A.M., Weetman, D.A., Sweeney, D. and Beeley, J.A. (1982). "Effect of extracellular polysaccharides on diffusion of NaF and C-14 labelled sucrose in human dental plaque and in sediments of the bacterium *Streptococcus sanguis* 804 (NCTC-10904)." Archives of Oral Biology **27**(11): 981-986.

Mellberg, J.R. (1992). "Hard-tissue substrates for evaluation of cariogenic and anti-cariogenic activity *in situ*." Journal of Dental Research **71**: 913-919.

Michels, P.A.M., Michels, J.P.J., Boonstra, J. and Konings, W.N. (1979). "Generation of an electrochemical proton gradient in bacteria by the excretion of metabolic end products." FEMS Microbiology Letters **5**(5): 357-364.

Mikx, F.H.M. and van der Hoeven, J.S. (1975). "Symbiosis of *Streptococcus mutans* and *Veillonella alcalescens* in mixed continuous cultures." Archives of Oral Biology **20**(7): 407-410.

Miller, M.B. and Bassler, B.L. (2001). "Quorum sensing in bacteria." Annual Review of Microbiology **55**: 165-199.

Miller, W.D. (1890). Micro-organisms of the human mouth. The S. S. White Dental Manufacturing. Philadelphia, PA, USA.

Milly, H., Festy, F., Watson, T.F., Thompson, I. and Banerjee, A. (2014). "Enamel white spot lesions can remineralise using bio-active glass and polyacrylic acid-modified bio-active glass powders." Journal of Dentistry **42**(2): 158-166.

Milnes, A.R., Bowden, G.H. and Hamilton, I.R. (1985). "Effect of NaF and pH on the growth and glycolytic rate of recently rate of recently isolated strains of oral *Lactobacillus* species." Journal of Dental Research **64**(3): 401-404.

Minah, G.E., Lovekin, G.B. and Finney, J.P. (1981). "Sucrose-induced ecological response of experimental dental plaques from caries-free and caries-suceptible human volunteers." Infection and Immunity **34**(3): 662-675.

Mitchell, P. (1966). "Chemiosmotic coupling in oxidative and photosynthetic phosphorylation." Biological Reviews of the Cambridge Philosophical Society **41**(3): 445-501.

Mittelman, M.W. (1996). Adhesion to biomaterials. Bacterial Adhesion: Molecular and Ecological Diversity. Fletcher, M. New York, Wiley-Liss**:** 89-127.

Miyazaki, H. and Morimoto, M. (1996). "Changes in caries prevalence in Japan." European Journal of Oral Sciences **104**(4): 452-458.

Mobley, M.J. (1981). "Fluoride uptake from *in situ* brushing with a SnF_2 and a NaF dentifrice." Journal of Dental Research **60**(12): 1943-1948.

Mohammed, N.R., Lynch, R.J.M. and Anderson, P. (2014). "Effects of fluoride concentration on enamel demineralization kinetics *in vitro*." Journal of Dentistry **42**(5): 613-618.

Molin, S. and Tolker-Nielsen, T. (2003). "Gene transfer occurs with enhanced efficiency in biofilms and induces enhanced stabilisation of the biofilm structure." Current Opinion in Biotechnology **14**(3): 255-261.

Monchois, V., Willemot, R.-M. and Monsan, P. (1999). "Glucansucrases: mechanism of action and structure–function relationships." FEMS Microbiology Reviews **23**(2): 131-151.

Monchois, V., Vignon, M., Escalier, P.-C., Svensson, B. and Russell, R.R.B. (2000). "Involvement of Gln937 of *Streptococcus downei* GTF-I glucansucrase in transition-state stabilization." European Journal of Biochemistry **267**(13): 4127-4136.

Morch, T., Punwani, I. and Greve, E. (1971). "The possible role of complex forming substances in the decalcification phase of the caries process." Caries Research **5**(2): 135-143.

Moreno, E.C. and Zahradnik, R.T. (1974). "Chemistry of enamel subsurface demineralisation *in vitro*." Journal of Dental Research **53**(2): 226-235.

Moses, J., Alexander, J. and Agus, M. (2009). "The correlation and level of agreement between end-tidal and blood gas pCO_2 in children with respiratory distress: a retrospective analysis." BMC Pediatrics **9**(1): 20.

Müller, H.-P. and Behbehani, E. (2005). "Methods for measuring agreement: glucose levels in gingival crevice blood." Clinical Oral Investigations **9**(1): 65-69.

Mulligan, A.M., Wilson, M. and Knowles, J.C. (2003). "Effect of increasing silver content in phosphate-based glasses on biofilms of *Streptococcus sanguis*." Journal of Biomedical Materials Research Part A **67A**(2): 401-412.

Murray, J.J., Rugg-Gunn, A.J. and Jenkins, G.N. (1991). Fluorides in caries prevention, Oxford: Wright

Murray, P.A., Levine, M.J., Tabak, L.A. and Reddy, M.S. (1982). "Specificity of salivary-bacterial interactions. 2. Evidence for a lectin on *Streptococcus sanguis* with specificity for a neuac-alpha-2,3GA1,-beta-1,3GA1NAC sequence." Biochemical and Biophysical Research Communications **106**(2): 390-396.

Murray, P.A., Levine, M.J., Reddy, M.S., Tabak, L.A. and Bergey, E.J. (1986). "Preparation of a sialic acid-binding protein from *Streptococcus mitis* KS32AR." Infection and Immunity **53**(2): 359-365.

Myrberg, N. (1968). Proton magnetic resonance in human dental enamel and dentine. An experimental investigation using wide line NMR. Transactions of the Royal Schools of Dentistry, Stockholm and Umea. Stockholm, Tandlakarhogskolan. **14:** 1-62.

Nealson, K.H. and Hastings, J.W. (1979). "Bacterial bioluminescence: its control and ecological significance." Microbiological Reviews **43**(4): 496-518.

Newbrun, E. (1983). Cariology, Baltimore: Williams & Wilkins

Newman, H.N. and Poole, D.F.G. (1974). "Observations with scanning and transmission electron-microscopy on structure of human surface enamel." Archives of Oral Biology **19**(12): 1135-1143.

NHS (2008). "NHS Report: NHS expenditure for general dental services and personal dental services, England 1997/98 to 2005/06." The NHS Information Centre for Health and Social Care, Dental Statistics.

NIH (2001). "Diagnosis and management of dental caries throughout life." NIH concensus statement **18**: 1-23.

Nobre dos Santos, M., Melo dos Santos, L., Francisco, S.B. and Cury, J.A. (2002). "Relationship among dental plaque composition, daily sugar exposure and caries in the primary dentition." Caries Research **36**(5): 347-352.

Nordström, A. and Birkhed, D. (2010). "Preventive effect of high-fluoride dentifrice (5,000 ppm) in caries-active adolescents: a 2-year clinical trial." Caries Research **44**(3): 323-331.

Nordström, A. and Birkhed, D. (2013). "Effect of a third application of toothpastes (1450 and 5000 ppm F), including a 'massage' method on fluoride retention and pH drop in plaque." Acta Odontologica Scandinavica **71**(1): 50-56.

Norwood, D.E. and Gilmour, A. (2000). "The growth and resistance to sodium hypochlorite of *Listeria monocytogenes* in a steady-state multispecies biofilm." Journal of Applied Microbiology **88**(3): 512-520.

Nyvad, B. and Fejerskov, O. (1987). "Scanning electron microscopy of early microbial colonisation of human enamel and root surfaces *in vivo*." Scandinavian Journal of Dental Research **95**(4): 287-296.

Nyvad, B. and Kilian, M. (1987). "Microbiology of the early colonisation of human-enamel and root surfaces *in vivo*." Scandinavian Journal of Dental Research **95**(5): 369-380.

Nyvad, B. and Fejerskov, O. (1989). "Structure of dental plaque and the plaque-enamel interface in human experimental caries." Caries Research **23**(3): 151-158.

Nyvad, B. and Kilian, M. (1990). "Comparison of the initial streptococcal microflora on dental enamel in caries-active and caries-inactive individuals." Caries Research **24**(4): 267-272.

Nyvad, B. (1993). "Microbial colonization of human tooth surfaces." APMIS. Supplementum **32**: 1-45.

Nyvad, B. and Fejerskov, O. (1994). Development, structure, and pH of dental plaque. Textbook of Clinical Cariology. Thylstrup, A. and Fejerskov, O. Copenhagen, Munksgaard**:** 89-110.

Nyvad, B., Crielaard, W., Mira, A., Takahashi, N. and Beighton, D. (2013). "Dental caries from a molecular microbiological perspective." Caries Research **47**(2): 89-102.

O'Reilly, J.E. (1975). "Fluorescence experiments with quinine." Journal of Chemical Education **52**(9): 610-612.

O'Reilly, M.M. and Featherstone, J.D.B. (1987). "De- and remineralization around orthodontic appliances: an *in vivo* study." American Journal of Orthodontics and Dentofacial Orthopedics **92**: 33-40.

Ogaard, B., Arends, J., Schuthof, J., Rolla, G. and Ekstrand, J. (1986a). "Action of fluoride on carious lesion development *in vivo*." Caries Research **20**(2): 175-175.

Ogaard, B., Arends, J., Schuthof, J., Rolla, G., Ekstrand, J. and Oliveby, A. (1986b). "Action of fluoride on initiation of early enamel caries *in vivo* - a microradiographical investigation." Caries Research **20**(3): 270-277.

Ogaard, B., Rølla, G. and Arends, J. (1988). "*In vivo* progress of enamel and root surface lesions under plaque as a function of time." Caries Research **22**(5): 302-305.

Ogaard, B. (2001). "CaF_2 formation: cariostatic properties and factors of enhancing the effect." Caries Research **35**: 40-44.

Onose, H. and Sandham, H.J. (1976). "pH changes during culture of human dental plaque streptococci on mitis-salivarius agar." Archives of Oral Biology **21**(5): 291-296.

Owens, G.J. (2013). *In vitro* caries: dental plaque formation and acidogenicity. PhD, University of Liverpool.

Pader, M., Cancro, L.P. and Guillo, B. (1977). "Evaluation of fluoride dentifrices." Journal of the Society of Cosmetic Chemists **28**(11): 681-694.

Paes Leme, A.F., Tabchoury, C.P.M., Zero, D.T. and Cury, J.A. (2003). "Effect of fluoridated dentifrice and acidulated phosphate fluoride application on early artificial carious lesions." American Journal of Dentistry **16**(2): 91-95.

Paes Leme, A.F., Dalcico, R., Tabchoury, C.P.M., Cury, A.A.D., Rosalen, P.L. and Cury, J.A. (2004). "*In situ* effect of frequent sucrose exposure on enamel demineralization and on plaque composition after APF application and F dentifrice use." Journal of Dental Research **83**(1): 71-75.

Paes Leme, A.F., Koo, H., Bellato, C.M., Bedi, G. and Cury, J.A. (2006). "The role of sucrose in cariogenic dental biofilm formation--new insight." Journal of Dental Research **85**(10): 878-887.

Page, D.J. (1991). "A study of the effect of fluoride delivered from solution and dentifrices on enamel demineralisation." Caries Research **25**(4): 251-255.

Palamara, J., Phakey, P.P., Rachinger, W.A. and Orams, H.J. (1986a). "Laminated zones in carious human dental enamel." Journal of Oral Pathology & Medicine **15**(2): 109-114.

Palamara, J., Phakey, P.P., Rachinger, W.A. and Orams, H.J. (1986b). "Ultrastructure of the intact surface zone of white spot and brown spot carious lesions in human-enamel." Journal of Oral Pathology & Medicine **15**(1): 28-35.

Palmer, R.J. and White, D.C. (1997). "Developmental biology of biofilms: implications for treatment and control." Trends in Microbiology **5**(11): 435-440.

Pandit, S., Kim, H.J., Song, K.Y. and Jeon, J.G. (2013). "Relationship between fluoride concentration and activity against virulence factors and viability of a cariogenic biofilm: *in vitro* study." Caries Research **47**(6): 539-547.

Parmly, L.S. (1819). A practical guide to the management of the teeth: comprising a discovery of the origin of caries, or decay of the teeth, with its prevention and cure. Philadelphia, Collins & Croft.

Patel, P.R. and Brown, W.E. (1975). "Thermodynamic solubility product of human tooth enamel - powdered sample." Journal of Dental Research **54**(4): 728-736.

Pecharki, G.D., Cury, J.A., Paes Leme, A.F., Tabchoury, C.P., Del Bel Cury, A.A., Rosalen, P.L. and Bowen, W.H. (2005). "Effect of sucrose containing iron (II) on dental biofilm and enamel demineralization *in situ*." Caries Research **39**(2): 123-129.

Percival, S.L., Malic, S., Cruz, H. and Williams, D.W. (2011). Introduction to biofilms. Biofilms and Veterinary Medicine. Percival, S.L., Knottenbelt, D.C. and Cochrane, C.A. New York, Springer. **6:** 41-68.

Pessan, J.P., Sicca, C.M., De Souza, T.S., Da Silva, S.M.B., Whitford, G.M. and Buzalaf, M.A.R. (2006). "Fluoride concentrations in dental plaque and saliva after the use of a fluoride dentifrice preceded by a calcium lactate rinse." European Journal of Oral Sciences **114**(6): 489-493.

Pessan, J.P., Silva, S.M.B., Lauris, J.R.P., Sampaio, F.C., Whitford, G.M. and Buzalaf, M.A.R. (2008). "Fluoride uptake by plaque from water and from dentifrice." Journal of Dental Research **87**(5): 461-465.

Pessan, J.P., Alves, K.M.R.P., Ramires, I., Taga, M.F.L., Sampaio, F.C., Whitford, G.M. and Buzalaf, M.A.R. (2010). "Effects of regular and low-fluoride dentifrices on plaque fluoride." Journal of Dental Research **89**(10): 1106-1110.

Peters, A.C. and Wimpenny, J.W.T. (1988). "A constant-depth laboratory model film fermentor." Biotechnology and Bioengineering **32**(3): 263-270.

Petersen, F.C., Tao, L. and Scheie, A.A. (2005). "DNA binding-uptake system: a link between cell-to-cell communication and biofilm formation." Journal of Bacteriology **187**(13): 4392-4400.

Petersen, P.E. (2003). "The World Oral Health Report 2003: continuous improvement of oral health in the 21st century – the approach of the WHO Global Oral Health Programme." Community Dentistry and Oral Epidemiology **31**: 3-24.

Petzold, M. (2001). "The influence of different fluoride compounds and treatment conditions on dental enamel: A descriptive *in vitro* study of the CaF_2 precipitation and microstructure." Caries Research **35**: 45-51.

Phillips, S.R., Wilson, L.J. and Borkman, R.F. (1986). "Acrylamide and iodide fluorescence quenching as a structural probe of tryptophan microenvironment in bovine lens crystallins." Current Eye Research **5**(8): 611-619.

Pincus, P. (1949). "Production of dental caries." British Medical Journal **2**: 358.

Plasschaert, A.J., Mörch, T. and König, K.G. (1972). "Effect of sodium lactate under conditions of neutral pH on the release of calcium from the enamel surface *in vitro*." Caries Research **6**(4): 334-345.

Pollard, M.A. (1995). "Potential cariogenicity of starches and fruits as assessed by the plaque-sampling method and an intraoral cariogenicity test." Caries Research **29**(1): 68-74.

Poolman, B., Knol, J., van der Does, C., Henderson, P.J.F., Liang, W.J., Leblanc, G., Pourcher, T. and Mus Veteau, I. (1996). "Cation and sugar selectivity determinants in a novel family of transport proteins." Molecular Microbiology **19**(5): 911-922.

Posner, A.S. and Perloff, A. (1957). "Apatites deficient in divalent cations." Journal of Research of the National Bureau of Standards **58**(5): 279-286.

Postma, P.W., Lengeler, J.W. and Jacobson, G.R. (1993). "Phosphoenolpyruvate: carbohydrate phosphotransferase systems of bacteria." Microbiological Reviews **57**(3): 543-594.

Pratten, J. (1998). The effect of chlorhexidine and other antimicrobial agents on the formation and viability of oral bacterial biofilms. PhD, University of London, Eastman Dental Institute for Oral Health Care Sciences.

Pratten, J., Barnett, P. and Wilson, M. (1998a). "Composition and susceptibility to chlorhexidine of multispecies biofilms of oral bacteria." Applied and Environmental Microbiology **64**(9): 3515-3519.

Pratten, J., Smith, A.W. and Wilson, M. (1998b). "Response of single species biofilms and microcosm dental plaques to pulsing with chlorhexidine." Journal of Antimicrobial Chemotherapy **42**(4): 453-459.

Pratten, J., Wills, K., Barnett, P. and Wilson, M. (1998c). "*In vitro* studies of the effect of antiseptic-containing mouthwashes on the formation and viability of *Streptococcus sanguis* biofilms." Journal of Applied Microbiology **84**(6): 1149-1155.

Pratten, J., Bedi, R. and Wilson, M. (1999). "Growth of microcosm plaques in the presence of milk and fluoridated milk." Journal of Dental Research **78**(5): 1069-1069.

Pratten, J. and Wilson, M. (1999). "Antimicrobial susceptibility and composition of microcosm dental plaques supplemented with sucrose." Antimicrobial Agents and Chemotherapy **43**(7): 1595-1599.

Pratten, J., Bedi, R. and Wilson, M. (2000). "An *in vitro* study of the effect of fluoridated milk on oral bacterial biofilms." Applied and Environmental Microbiology **66**(4): 1720-1723.

Pratten, J., Wilson, M. and Spratt, D.A. (2003). "Characterization of *in vitro* oral bacterial biofilms by traditional and molecular methods." Oral Microbiology and Immunology **18**(1): 45-49.

Pratten, J. (2007). "Growing oral biofilms in a constant depth film fermentor (CDFF)." Current Protocols in Microbiology: 1B.5.1-1B.5.17.

Pratten, J. and Ready, D. (2010). Use of Biofilm Model Systems to Study Antimicrobial Susceptibility. Antibiotic Resistance Protocols, Second Edition. Gillespie, S.H. and McHugh, T.D. Totowa, Humana Press Inc. **642:** 203-215.

Pretty, I.A., Edgar, W.M. and Higham, S.M. (2004). "The validation of quantitative light-induced fluorescence to quantify acid erosion of human enamel." Archives of Oral Biology **49**(4): 285-294.

Pretty, I.A., Edgar, W.M., Smith, P.W. and Higham, S.M. (2005). "Quantification of dental plaque in the research environment." Journal of Dentistry **33**(3): 193-207.

Prinz, H. (1909). A history of dentistry. Philadelphia, Lea & Febiger.

Pritchard, P.H. and Bourquin, A.W. (1984). The use of microcosms for evaluation of interactions between pollutants and microorganisms. Advances in Microbial Ecology. Marshall, K.C.**:** 133-215.

Psarros, N., Feige, U. and Duschner, H. (1990). "Interactions of micromolar concentrations of fluoride with *Streptococcus rattus* FA-1." Caries Research **24**(3): 189-197.

Queiroz, C.S., Hara, A.T., Paes Leme, A.F. and Cury, J.A. (2008). "pH-cycling models to evaluate the effect of low fluoride dentifrice on enamel de- and remineralization." Brazilian Dental Journal **19**(1): 21-27.

Quivey, R.G., Kuhnert, W.L. and Hahn, K. (2000). Adaptation of oral streptococci to low pH. Advances in Microbial Physiology, Academic Press. **Volume 42:** 239-274.

Ramage, G., Martinez, J.P. and Lopez-Ribot, J.L. (2006). "*Candida* biofilms on implanted biomaterials: a clinically significant problem." FEMS Yeast Research **6**(7): 979-986.

Ramage, G., Culshaw, S., Jones, B. and Williams, C. (2010). "Are we any closer to beating the biofilm: novel methods of biofilm control." Current Opinion in Infectious Diseases **23**(6): 560-566.

Ramasubbu, N., Paloth, V., Luo, Y.G., Brayer, G.D. and Levine, M.J. (1996). "Structure of human salivary alpha-amylase at 1.6 angstrom resolution: implications for its role in the oral cavity." Acta Crystallographica Section D - Biological Crystallography **52**: 435-446.

Reed, J., Troke, J.J., Schmit, J., Han, S., Teitell, M.A. and Gimzewski, J.K. (2008). "Live cell interferometry reveals cellular dynamism during force propagation." ACS Nano **2**(5): 841-846.

Rehm, D. and Weller, A. (1970). "Kinetics of fluorescence quencing by electron and H-atom transfer." Israel Journal of Chemistry **8**(2): 259-271.

Reizer, J., Reizer, A. and Saier, M.H., Jr. (1994). "A functional superfamily of sodium/solute symporters." Biochimica et Biophysica Acta **1197**(2): 133-166.

Renz, C.L. and Bibby, B.G. (1989). "*In vitro* acid production from starch and sucrose in saliva." ASDC Journal of Dentistry for Children **56**(4): 267-269.

Reynolds, E.C. (2008). "Calcium phosphate-based remineralization systems: scientific evidence?" Australian Dental Journal **53**(3): 268-273.

Ribeiro, C.C., Tabchoury, C.P., Del Bel Cury, A.A., Tenuta, L.M., Rosalen, P.L. and Cury, J.A. (2005). "Effect of starch on the cariogenic potential of sucrose." British Journal of Nutrition **94**(1): 44-50.

Richards, M.P. (2002). "A brief review of the archaeological evidence for Palaeolithic and Neolithic subsistence." European Journal of Clinical Nutrition **56**(12): 16 p following 1262.

Ricketts, D.N.J., Kidd, E.A.M. and Beighton, D. (1995). "Operative and microbiological validation of visual, radiographic and electronic diagnosis of occlusal caries in non-cavitated teeth judged to be in need of operative care." British Dental Journal **179**(6): 214-220.

Ring, M.E. (1971). "Anton van Leeuwenhoek and the tooth-worm." Journal of the American Dental Association **83**(5).

Ritz, H.L. (1967). "Microbial population shifts in developing human dental plaque." Archives of Oral Biology **12**(12): 1561-1568.

Roberts, A.P. and Mullany, P. (2010). "Oral biofilms: a reservoir of transferable, bacterial, antimicrobial resistance." Expert Review of Anti-infective Therapy **8**(12): 1441-1450.

Robertson, W. (1835). A practical treatise on the diseases of the teeth. London, Longman, Rees, Orme, Brown, Green and Longman.

Robinson, C., Weatherell, J.A. and Hallsworth, A.S. (1971). "Variation in composition of dental enamel within thin ground tooth sections." Caries Research **5**(1): 44-57.

Robinson, C., Weatherell, I.A. and Hallsworth, A.S. (1983). Alterations in the composition of permanent human enamel during carious attack. Demineralisation and Remineralisation of the Teeth. Leach, S.A. and Edgar, W.M. Oxford, IRL Press**:** 209-223.

Robinson, C., Shore, R.C., Brookes, S.J., Strafford, S., Wood, S.R. and Kirkham, J. (2000). "The chemistry of enamel caries." Critical Reviews in Oral Biology & Medicine **11**(4): 481-495.

Robinson, C. (2009). "Fluoride and the caries lesion: interactions and mechanism of action." European Archives of Paediatric Dentistry **10**(3): 136-140.

Rolla, G. (1989). "Why is sucrose so cariogenic? The role of glucosyltransferase and polysaccharides." Scandinavian Journal of Dental Research **97**(2): 115-119.

Rolla, G. and Saxegaard, E. (1990). "Critical evaluation of the composition and use of topical fluorides, with emphasis on the role of calcium fluoride in caries inhibition." Journal of Dental Research **69 Spec No**: 780-785; discussion 820-783.

Rose, R.K., Dibdin, G.H. and Shellis, R.P. (1993). "A quantitative study of calcium-binding and aggregation in selected oral bacteria." Journal of Dental Research **72**(1): 78-84.

Rose, R.K., Shellis, R.P. and Lee, A.R. (1996). "The role of cation bridging in microbial fluoride binding." Caries Research **30**(6): 458-464.

Rosenberg, M. and Kjelleberg, S. (1986). "Hydrophobic interactions - role in bacterial adhesion." Advances in Microbial Ecology **9**: 353-393.

Rousseau, C., Vaidya, S., Creanor, S.L., Hall, A.F., Girkin, J.M., Whitters, C.J., Strang, R. and McHugh, S. (2002). "The effect of dentine on fluorescence measurements of enamel lesions *in vitro*." Caries Research **36**(6): 381-385.

Rugg-Gunn, A.J. and Hackett, A.F. (1993). Nutrition and dental health, Oxford: Oxford University Press.

Russell, R.R., Aduse-Opoku, J., Sutcliffe, I.C., Tao, L. and Ferretti, J.J. (1992). "A binding protein-dependent transport system in *Streptococcus mutans* responsible for multiple sugar metabolism." Journal of Biological Chemistry **267**(7): 4631-4637.

Russell, R.R.B. (2008). "How has genomics altered our view of caries microbiology?" Caries Research **42**(5): 319-327.

Russell, R.R.B. (2009). Bacterial Polysaccharides: Current Innovations and Future Trends. Ullrich, M. Norfolk, UK, Caister Academic Press.

Rutter, P.R. and Vincent, B. (1980). The adhesion of microrganisms to surfaces: physico-chemical aspects. Microbial Adhesion to Surfaces. Berkley, R.C.W., Lynch, J.M., Melling, J., Rutter, P.R. and Vincent, B. Chichester, Ellis Horwood**:** 79-93.

Sacksteder, L.A., Ballew, R.M., Brown, E.A., Demas, J.N., Nesselrodt, D. and Degraff, B.A. (1990). "Photophysics in a disco - luminscence quenching of quinine." Journal of Chemical Education **67**(12): 1065-1067.

Salmon, J.M., Kohen, E., Viallet, P., Hirschberg, J.G., Wouters, A.W., Kohen, C. and Thorell, B. (1982). "Microspectrofluorometric approach to ther study of free bound NAD(P)H ratio as metabolic indicator in various cell types." Photochemistry and Photobiology **36**(5): 585-593.

Sandham, H.J. and Kleinberg, I. (1969). "Effect of fluoride on interrelation between glucose utilisation pH and carbohydrate storage in a salivary sediment system." Archives of Oral Biology **14**(6): 619-628.

Scannapieco, F.A., Bergey, E.J., Reddy, M.S. and Levine, M.J. (1989). "Characterisation of salivary alpha-amylase binding to *streptococcus sanguis*." Infection and Immunity **57**(9): 2853-2863.

Schachtele, C., Harlander, S. and Germaine, G. (1977). "*Streptococcus mutans* dextransucrase activities producing water-soluble and water-insoluble glucans." Journal of Dental Research **56**: A133-A133.

Schachtele, C.F. and Jensen, M.E. (1982). "Comparison of methods for monitoring changes in the pH of human dental plaque." Journal of Dental Research **61**(10): 1117-1125.

Schamschula, R.G., Sugar, E., Un, P.S.H., Toth, K., Barmes, D.E. and Adkins, B.L. (1985). "Physiological indicators of fluoride exposure and utilisation - an epidemiological study." Community Dentistry and Oral Epidemiology **13**(2): 104-107.

Schatz, A. and Martin, J.J. (1955). "Speculation on lactobacilli and acids as possible anticaries factors." N.Y. State Dental Journal **21**: 367-379.

Scheie, A.A., Eggen, K.H. and Rölla, G. (1987). "Glucosyltransferase activity in human *in vivo* formed enamel pellicle and in whole saliva." Scandinavian Journal of Dental Research **95**(3): 212-215.

Scheie, A.A., Fejerskov, O., Lingstrom, P., Birkhed, D. and Manji, F. (1992). "Use of palladium touch microelectrodes under field conditions for *in vivo* assessment of dental plaque pH in children." Caries Research **26**(1): 44-52.

Schilling, K.M. and Bowen, W.H. (1988). "The activity of glucosyltransferase adsorbed onto saliva-coated hydroxyapatite." Journal of Dental Research **67**(1): 2-8.

Schilling, K.M. and Bowen, W.H. (1992). "Glucans synthesized *in situ* in experimental salivary pellicle function as specific binding sites for *Streptococcus mutans*." Infection and Immunity **60**(1): 284-295.

Schupbach, P., Guggenheim, B. and Lutz, F. (1990). "Human root caries - histopathology of advanced lesions." Caries Research **24**(3): 145-158.

Selwyn, J.E. and Steinfeld, J.I. (1969). "Collision-induced predissociation by van der Waals' interaction." Chemical Physics Letters **4**(4): 217-220.

Shah, H.N., Bonnett, R., Mateen, B. and Williams, R.A. (1979). "The porphyrin pigmentation of subspecies of *Bacteroides melaninogenicus*." Biochemical Journal **180**(1): 45-50.

Shah, H.N. and Collins, D.M. (1990). "NOTES: *Prevotella*, a new genus to include *Bacteroides melaninogenicus* and related species formerly classified in the genus *Bacteroides*." International Journal of Systematic Bacteriology **40**(2): 205-208.

Sharma, M., Dhillon, A.S. and Newbrun, E. (1974). "Cell-bound glucosyltransferase activity of *Streptococcus sanguis* strain 804." Archives of Oral Biology **19**(11): 1063-1072.

Shaw, J.H. (1983). "The role of sugar in the aetiology of dental caries. 6. Evidence from experimental animal research." Journal of Dentistry **11**(3): 209-213.

Sheiham, A. (2001). "Dietary effects on dental diseases." Public Health Nutrition **4**(2b): 569-591.

Shellis, R.P. and Dibdin, G.H. (1988). "Analysis of the buffering systems in dental plaque." Journal of Dental Research **67**(2): 438-446.

Shemesh, M., Tam, A., Aharoni, R. and Steinberg, D. (2010). "Genetic adaptation of *Streptococcus mutans* during biofilm formation on different types of surfaces." BMC Microbiology **10**.

Shi, X.Q., Tranaeus, S. and Angmar-Mansson, B. (2001). "Comparison of QLF and DIAGNOdent for quantification of smooth surface caries." Caries Research **35**(1): 21-26.

Shibata, S., Nagata, K., Nakamura, R., Tsunemitsu, A. and Misaki, A. (1980). "Interaction of parotid-saliva basic glycoprotein with *Streptococcus sanguis* ATCC-10557." Journal of Periodontology **51**(9): 499-504.

Shields, C.P., Moss, M.E., Featherstone, J.D.B., Proskin, H.M. and Billings, R.J. (1995). "Chemical analysis of saliva - a longitudinal study." Journal of Dental Research **74**: 15-15.

Shields, C.P., Moss, M.E., Billings, R.J. and Featherstone, J.D.B. (1997). "A longitudinal chemical analysis of saliva." Journal of Dental Research **76**: 177-177.

Shigetani, Y., Takenaka, S., Okamoto, A., Abu-Bakr, N., Iwaku, M. and Okiji, T. (2008). "Impact of *Streptococcus mutans* on the generation of fluorescence from artificially induced enamel and dentin carious lesions *in vitro*." Odontology **96**(1): 21-25.

Shimura, N. and Onisi, M. (1978). "Effect of NaF on bacterial production of polysaccharide and subsequent adsoprtion on hydroxyapatite." Journal of Dental Research **57**(9-10): 928-931.

Shiota, T. (1956). "Effect of sodium fluoride on oral *Lactobacilli* isolated from the rat." Journal of Dental Research **35**(6): 939-946.

Silverstone, L. (1982). "Relationship of the macroscopic, histological and radiographic appearance of interproximal lesions in human teeth: *in vitro* study using artificial caries technique." Journal of Paediatric Dentistry **3**: 414–422.

Silverstone, L.M. (1970). "The histopathology of early approximal caries in the enamel of primary teeth." ASDC Journal of Dentistry for Children **37**(3): 201-210.

Silverstone, L.M. and Johnson, N.W. (1971). "The effect on sound human enamel of exposure to calcifying fluids *in vitro*." Caries Research **5**(4): 323-342.

Silverstone, L.M., Saxton, C.A., Dogon, I.L. and Fejerskov, O. (1975). "Variation in the pattern of acid etching of human dental enamel examined by scanning electron microscopy." Caries Research **9**(5): 373-387.

Sissons, C.H., Hancock, E.M., Perinpanayagam, H.E.R. and Cutress, T.W. (1988). "The bacteria responsible for ureolysis in artificial dental plaque." Archives of Oral Biology **33**(10): 727-733.

Sissons, C.H. and Yakub, S. (2000). "Suppression of urease levels in *Streptococcus salivarius* by cysteine, related compounds and by sulfide." Oral Microbiology and Immunology **15**(5): 317-324.

Skillman, L.C., Sutherland, I.W. and Jones, M.V. (1998). "The role of exopolysaccharides in dual species biofilm development." Journal of Applied Microbiology **85**: 13S.

Socransky, S.S. and Haffajee, A.D. (2002). "Dental biofilms: difficult therapeutic targets." Periodontology 2000 **28**(1): 12-55.

Sollböhmer, O., May, K.P. and Anders, M. (1995). "Force microscopical investigation of human teeth in liquids." Thin Solid Films **264**(2): 176-183.

Soni, N.N. and Brudevold, F. (1959). "Microradiographic and polarized light studies of initial carious lesions." Journal of Dental Research **38**: 1187-1194.

Sorvari, R., Pelttari, A. and Meurman, J.H. (1996). "Surface ultrastructure of rat molar teeth after experimentally induced erosion and attrition." Caries Research **30**(2): 163-168.

Spatafora, G., Rohrer, K., Barnard, D. and Michalek, S. (1995). "A *Streptococcus mutans* mutant that synthesizes elevated levels of intracellular polysaccharide is hypercariogenic *in vivo*." Infection and Immunity **63**(7): 2556-2563.

Speiser, S., van der Werf, R. and Kommandeur, J. (1973). "Photoquenching: the dependence of the primary quantum view of a monophotonic laser-induced photochemical process on the intensity and duration of the exciting pulse." Chemical Physics **1**(4): 297-305.

Spitzer, D. and Bosch, J.J. (1976). "The total luminescence of bovine and human dental enamel." Calcified Tissue Research(2): 201-208.

Spoering, A.L. and Gilmore, M.S. (2006). "Quorum sensing and DNA release in bacterial biofilms." Current Opinion in Microbiology **9**(2): 133-137.

Sreebny, L.M. (1982). "Sugar availability, sugar consumption and dental caries." Community Dentistry and Oral Epidemiology **10**(1): 1-7.

Steensels, D., Verhaegen, J. and Lagrou, K. (2011). "Matrix-assisted laser desorption ionization-time of flight mass spectrometry for the identification of bacteria and yeasts in a clinical microbiological

laboratory: a review." Acta Clinica Belgica: International Journal of Clinical and Laboratory Medicine **66**(4): 267-273.

Stephan, R.M. (1940). "Changes in hydrogen ion concentration on tooth surfaces and in carious lesions." Journal of the American Dental Association **27**: 718.

Stephan, R.M. (1944). "Intra-oral hydrogen-ion concentrations associated with dental caries activity." Journal of Dental Research **23**(4): 257-266.

Stewart, P.S., Camper, A.K., Handran, S.D., Huang, C.T. and Warnecke, M. (1997). "Spatial distribution and coexistence of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* in biofilms." Microbial Ecology **33**(1): 2-10.

Stickland, L.H. (1934). "Studies in the metabolism of the strict anaerobes (genus *Clostridium*): The chemical reactions by which *Cl. sporogenes* obtains its energy." Biochemical Journal **28**(5): 1746-1759.

Stoodley, P., Boyle, J.D., Dodds, I. and Lappin-Scott, H.M. (1997). Consensus model of biofilm structure. Biofilms: Community Interactions and Control. Third meeting of the British Biofilm Club, Gregynog Hall, Powys, 26-28 September 1997**:** 1-9.

Stookey, G.K., Schemehorn, B.R., Cheetham, B.L., Wood, G.D. and Walton, G.V. (1985). "*In situ* fluoride uptake from fluoride dentifrices by carious enamel." Journal of Dental Research **64**(6): 900-903.

Stookey, G.K. (2004). "Optical methods--quantitative light fluorescence." Journal of Dental Research **83 Spec No C**: C84-88.

Stookey, G.K., Featherstone, J.D.B., Rapozo-Hilo, M., Schemehorn, B.R., Williams, R.A., Baker, R.A., Barker, M.L., Kaminski, M.A., McQueen, C.M., Amburgey, J.S., Casey, K. and Faller, R.V. (2011). "The Featherstone laboratory pH cycling model: a prospective, multi-site validation exercise." American Journal of Dentistry **24**(5): 322-328.

Stromberg, N. and Boren, T. (1992). "*Actinomyces* tissue-specificity may depend on differences in receptor specificity for galnac-beta-containing glycoconjugates." Infection and Immunity **60**(8): 3268-3277.

Stromberg, N., Boren, T., Carlen, A. and Olsson, J. (1992). "Salivary receptors for galnac-beta-sensitive adherence of *Actinomyces* spp. - evidence for heterogeneous galnac-beta and proline-rich protein receptor properties." Infection and Immunity **60**(8): 3278-3286.

Stubel, H. (1911). "The fluorescence of animal tissues in ultraviolet light." Pflugers Archiv Fur Die Gesamte Physiologie Des Menschen Und Der Tiere **142**(1/2): 1-14.

Sturr, M.G. and Marquis, R.E. (1992). "Comparative acid tolerances and inhibitor sensitivities of isolated F-ATPases of oral lactic acid bacteria." Applied and Environmental Microbiology **58**(7): 2287-2291.

Suddick, R.P. and Harris, N.O. (1990). "Historical perspectives of oral biology: a series." Critical Reviews in Oral Biology & Medicine **1**(2): 135-151.

Sundstrom, F., Fredriksson, K., Montan, S., Hafstrombjorkman, U. and Strom, J. (1985). "Laser-induced fluorescence from sound and carious tooth substance - spectroscopic studies." Swedish Dental Journal **9**(2): 71-80.

Sutton, N.A., Hughes, N. and Handley, P.S. (1994). "A comparison of conventional SEM techniques, low temperature SEM and the electroscan wet scanning electron microscope to study the structure of a biofilm of *Streptococcus crista* CR3." Journal of Applied Bacteriology **76**(5): 448-454.

Sutton, S.V.W., Bender, G.R. and Marquis, R.E. (1987). "Fluoride inhibition of proton-translocating ATPases of oral bacteria." Infection and Immunity **55**(11): 2597-2603.

Svensater, G., Welin, J., Wilkins, J.C., Beighton, D. and Hamilton, I.R. (2001). "Protein expression by planktonic and biofilm cells of *Streptococcus mutans*." FEMS Microbiology Letters **205**(1): 139-146.

Tada, A., Senpuku, H., Motozawa, Y., Yoshihara, A., Hanada, N. and Tanzawa, H. (2006). "Association between commensal bacteria and opportunistic pathogens in the dental plaque of elderly individuals." Clinical Microbiology and Infection **12**(8): 776-781.

Takahashi, N. and Nyvad, B. (2008). "Caries ecology revisited: microbial dynamics and the caries process." Caries Research **42**(6): 409-418.

Takahashi, N. and Nyvad, B. (2011). "The role of bacteria in the caries process: ecological perspectives." Journal of Dental Research **90**(3): 294-303.

Takahashi, N. and Washio, J. (2011). "Metabolomic effects of xylitol and fluoride on plaque biofilm *in vivo*." Journal of Dental Research **90**(12): 1463-1468.

Tanzer, J., Livingston, J. and Thompson, A. (2001). "The microbiology of primary dental caries in humans." Journal of Dental Education **65**(10): 1028-1037.

Tanzer, J.M., Krichevsky, M.I. and Keyes, P.H. (1969). "The metabolic fate of glucose catabolized by a washed stationary phase caries-conducive streptococcus." Caries Research **3**(2): 167-177.

Tanzer, J.M., Freedman, M.L., Woodiel, F.N., Eifert, R.L. and Rinehimer, L.A. (1976). "Association of *Streptococcus mutans* virulence with synthesis of intracellular polysaccharide." Journal of Dental Research **55**: B173-B173.

Tanzer, J.M. (1989). "On changing the cariogenic chemistry of coronal plaque." Journal of Dental Research **68**: 1576-1587.

Tao, L., Sutcliffe, I.C., Russell, R.R. and Ferretti, J.J. (1993a). "Transport of sugars, including sucrose, by the MSM transport system of *Streptococcus mutans*." Journal of Dental Research **72**(10): 1386-1390.

Tao, L., Sutcliffe, I.C., Russell, R.R. and Ferretti, J.J. (1993b). "Cloning and expression of the multiple sugar metabolism (*msm*) operon of *Streptococcus mutans* in heterologous streptococcal hosts." Infection and Immunity **61**(3): 1121-1125.

Tehrani, A., Brudevold, F., Attarzadeh, F., Vanhoute, J. and Russo, J. (1983). "Enamel demineralisation by mouthrinses containing different concentrations of sucrose." Journal of Dental Research **62**(12): 1216-1217.

ten Bosch, J.J. and Angmar-Månsson, B. (1991). "Invited review: A review of quantitative methods for studies of mineral content of intra-oral incipient caries lesions." Journal of Dental Research **70**(1): 2-14.

ten Bosch, J.J. (1996). Light scattering and related methods in caries diagnosis. Early Detection of Dental Caries: Proceedings of the 1st Annual Indiana Conference. Stookey, G.K. Indianapolis, IN, USA, Indiana University School of Dentistry**:** 81-90.

ten Bosch, J.J. (2000). Summary of research of Quantitative Light-induced Fluorescence. Presented at early detection of dental caries II. Proceedings of the 4th Annual Indiana Conference. Indianapolis, USA.

ten Cate, J.M. and Duijsters, P.P.E. (1982). "Alternating demineralization and remineralization of artificial enamel lesions." Caries Research **16**(3): 201-210.

ten Cate, J.M. and Duijsters, P.P.E. (1983). "Influence of fluoride in solution on tooth demineralization." Caries Research **17**(3): 193-199.

ten Cate, J.M. (1990). "*In vitro* studies on the effects of fluoride on de- and remineralization." Journal of Dental Research **69**: 614-619.

ten Cate, J.M. and Featherstone, J.D.B. (1991). "Mechanistic aspects of the interactions between fluoride and dental enamel." Critical Reviews in Oral Biology & Medicine **2**: 283-296.

ten Cate, J.M. (1993). "The caries preventive effect of a fluoride dentifrice containing triclosan and zinc citrate, a compilation of *in vitro* and *in situ* studies." International Dental journal **43**(4 Suppl 1): 407-413.

ten Cate, J.M. (1997). "Review on fluoride, with special emphasis on calcium fluoride mechanisms in caries prevention." European Journal of Oral Sciences **105**(5 Pt 2): 461-465.

ten Cate, J.M., Damen, J.J.M. and Buijs, M.J. (1998). "Inhibition of dentin demineralization by fluoride *in vitro*." Caries Research **32**(2): 141-147.

ten Cate, J.M. (1999). "Current concepts on the theories of the mechanism of action of fluoride." Acta Odontologica Scandinavica **57**(6): 325-329.

ten Cate, J.M. (2001). "Remineralization of caries lesions extending into dentin." Journal of Dental Research **80**(5): 1407-1411.

ten Cate, J.M. (2006). "Biofilms, a new approach to the microbiology of dental plaque." Odontology **94**: 1-9.

ten Cate, J.M., Exterkate, R.A.M. and Buijs, M.J. (2006). "The relative efficacy of fluoride toothpastes assessed with pH cycling." Caries Research **40**(2): 136-141.

ten Cate, J.M., Larsen, M.J., Pearce, E.I.F. and Fejerskov, O. (2008). Chemical interactions between the tooth and oral fluids. Dental Caries: the Disease and its Clinical Management. Fejerskov, O. and Kidd, E.A.M. Oxford, Wiley-Blackwell**:** 209-232.

Tenuta, L.M.A., Del Bel Cury, A.A., Bortolin, M.C., Vogel, G.L. and Cury, J.A. (2006). "Ca, Pi, and F in the fluid of biofilm formed under sucrose." Journal of Dental Research **85**(9): 834-838.

Tenuta, L.M.A. and Cury, J.A. (2013). Laboratory and human studies to estimate anticaries efficacy of fluoride toothpastes. Toothpastes. VanLoveren, C. **23:** 108-124.

Theilade, E. (1986). "The non-specific theory in microbial etiology of inflammatory periodontal-diseases." Journal of Clinical Periodontology **13**(10): 905-911.

Theocharopoulos, A., Zou, L., Hill, R. and Cattell, M. (2010). "Wear quantification of human enamel and dental glass–ceramics using white light profilometry." Wear **269**(11–12): 930-936.

Thomas, C.M. and Nielsen, K.M. (2005). "Mechanisms of, and barriers to, horizontal gene transfer between bacteria." Nature Reviews Microbiology **3**(9): 711-721.

Thomas, R.Z., van der Mei, H.C., van der Veen, M.H., de Soet, J.J. and Huysmans, M.C.D.N.J.M. (2008). "Bacterial composition and red fluorescence of plaque in relation to primary and secondary caries next to composite: an *in situ* study." Oral Microbiology and Immunology **23**(1): 7-13.

Thomas, T.R. (1982a). "What's significant in roughness." Precision Engineering-Journal of the American Society for Precision Engineering **4**(2): 59-59.

Thomas, T.R. (1982b). "Defining the micro-topography of surfaces in thermal contact." Wear **79**(1): 73-82.

Thurnheer, T., Gmur, R., Shapiro, S. and Guggenheim, B. (2003). "Mass transport of macromolecules within an *in vitro* model of supragingival plaque." Applied and Environmental Microbiology **69**(3): 1702-1709.

Thurnheer, T., van der Ploeg, J.R., Giertsen, E. and Guggenheim, B. (2006). "Effects of *Streptococcus mutans gtfC* deficiency on mixed oral biofilms *in vitro*." Caries Research **40**(2): 163-171.

Thylstrup, A., Bruun, C. and Holmen, L. (1994). "*In vivo* caries models: mechanisms for caries initiation and arrestment." Advances in Dental Research **8**(2): 144-157.

Tomazic, B., Tomson, M. and Nancollas, G.H. (1975). "Growth of calcium phosphates on hydroxyapatite crystals - effect of magnesium." Archives of Oral Biology **20**(12): 803-808.

Toverud, G. (1957). "The influence of war and post-war conditions on the teeth of norwegian school-children. 3. Discussion of food-supply and dental condition in Norway and other European countries." Milbank Memorial Fund Quarterly-Health and Society **35**(4): 373-459.

Treasure, P. (1981). "Effects of fluoride, lithium and strontium on extracellular polysaccharide production by *Streptococcus mutans* and *Actinomyces viscosus*." Journal of Dental Research: 1601-1610.

Trombe, M.C., Clave, C. and Manias, J.M. (1992). "Calcium regulation of growth and differentiation in *Streptococcus pneumoniae*." Journal of General Microbiology **138**: 77-84.

Tronstad, L., Andreasen, J.O., Hasselgren, G., Kristerson, L. and Riis, I. (1981). "pH changes in dental tissues after root canal filling with calcium hydroxide." Journal of Endodontics **7**(1): 17-21.

Tsuneda, S., Aikawa, H., Hayashi, H., Yuasa, A. and Hirata, A. (2003). "Extracellular polymeric substances responsible for bacterial adhesion onto solid surface." FEMS Microbiology Letters **223**(2): 287-292.

Twetman, S., Axelsson, S., Dahlgren, H., Holm, A.K., Kallestal, C., Lagerlof, F., Lingstrom, P., Mejare, I., Nordenram, G., Norlund, A., Petersson, L.G. and Soder, B. (2003). "Caries-preventive effect of fluoride toothpaste: a systematic review." Acta Odontologica Scandinavica **61**(6): 347-355.

Twetman, S. (2009). "Caries prevention with fluoride toothpaste in children: an update." European Archives of Paediatric Dentistry **10**(3): 162-167.

Tyler, J.E. and Poole, D.F.G. (1984). "Uptake of fluoride by human enamel from ammonium bifluoride and consequent reduction in the penetration *in vitro* by caries-like lesions." Archives of Oral Biology **29**(12): 971-974.

Uehara, Y., Kikuchi, K., Nakamura, T., Nakama, H., Agematsu, K., Kawakami, Y., Maruchi, N. and Totsuka, K. (2001). "H_2O_2 produced by viridans group streptococci may contribute to inhibition of methicillin-resistant *Staphylococcus aureus* colonization of oral cavities in newborns." Clinical Infectious Diseases **32**(10): 1408-1413.

Vadeboncoeur, C. and Pelletier, M. (1997). "The phosphoenolpyruvate:sugar phosphotransferase system of oral streptococci and its role in the control of sugar metabolism." FEMS Microbiology Letters **19**(3): 187-207.

Valappil, S.P. and Higham, S.M. (2014). "Antibacterial effect of gallium and silver on *Pseudomonas aeruginosa* treated with gallium-silver-phosphate-based glasses." Bio-Medical Materials and Engineering **24**(3): 1589-1594.

Valappil, S.P., Owens, G.J., Miles, E.J., Farmer, N.L., Cooper, L., Miller, G., Clowes, R., Lynch, R.J.M. and Higham, S.M. (2014). "Effect of gallium on growth of *Streptococcus mutans* NCTC 10449 and dental tissues." Caries Research **48**(2): 137-146.

van Amerongen, J.P., Penning, C., Kidd, E.A.M. and ten Cate, J.M. (1992). "An *in vitro* assessment of the extent of caries under small occlusal cavities." Caries Research **26**(2): 89-93.

van der Hoeven, J.S. and Franken, H.C.M. (1984). "Effect of fluoride on growth and acid production by *Streptococcus mutans* in dental plaque." Infection and Immunity **45**(2): 356-359.

van der Veen, M.H., Ando, M., Stookey, G.K. and de Josselin de Jong, E. (2002). "A Monte Carlo simulation of the influence of sound enamel scattering coefficient on lesion visibility in light-induced fluorescence." Caries Research **36**(1): 10-18.

van der Veen, M.H., Thomas, R.Z., Huysmans, M.C.D.N.J.M. and de Soet, J.J. (2006). "Red autofluorescence of dental plaque bacteria." Caries Research **40**(6): 542-545.

van Houte, J., Winkler, K.C. and Jansen, H.M. (1969). "Iodophilic polysaccharide synthesis, acid production and growth in oral streptococci." Archives of Oral Biology **14**(1): 45-61.

van Houte, J. and Green, D.B. (1974). "Relationship between the concentration of bacteria in saliva and the colonization of teeth in humans." Infection and Immunity **9**(4): 624-630.

van Houte, J. (1980). "Bacterial specificity in the etiology of dental caries." International Dental Journal **30**(4): 305-326.

van Houte, J. and Russo, J. (1986). "Variable colonisation by oral streptococci in molar fissures of mono-infected gnotobiotic-rats." Infection and Immunity **52**(2): 620-622.

van Houte, J., Russo, J. and Prostak, K.S. (1989). "Increased pH-lowering ability of *Streptococcus mutans* cell masses associated with extracellular glucan-rich matrix material and the mechanisms involved." Journal of Dental Research **68**(3): 451-459.

van Houte, J. (1994). "Role of micro-organisms in caries etiology." Journal of Dental Research **73**(3): 672-681.

van Loveren, C., Fielmich, A.M. and Tenbrink, B. (1987). "Comparison of the effects of fluoride and the ionophore nigericin on acid production by *Streptococcus mutans* and the resultant *in vitro* enamel demineralisation." Journal of Dental Research **66**(11): 1658-1662.

van Veen, S.Q., Claas, E.C.J. and Kuijper, E.J. (2010). "High-throughput identification of bacteria and yeast by Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry in conventional medical microbiology laboratories." Journal of Clinical Microbiology **48**(3): 900-907.

Vicaretti, J., Thibodeau, E., Bender, G. and Marquis, R.E. (1984). "Reversible fluoride uptake and release by *Streptococcus mutans* GS-5 and FA-1." Current Microbiology **10**(6): 317-322.

Vieira, A.E.D., Delbem, A.C.B., Sassaki, K.T., Rodrigues, E., Cury, J.A. and Cunha, R.F. (2005). "Fluoride dose response in pH-cycling models using bovine enamel." Caries Research **39**(6): 514-520.

Vogel, G.L., Carey, C.M. and Ekstrand, J. (1992a). "Distribution of fluoride in saliva and plaque fluid after a 0.048 mol/L NaF rinse." Journal of Dental Research **71**(9): 1553-1557.

Vogel, G.L., Mao, Y., Carey, C.M., Chow, L.C. and Takagi, S. (1992b). "*In vivo* fluoride concentrations measured for two hours after a NaF or a novel two-solution rinse." Journal of Dental Research **71**(3): 448-452.

Vogel, G.L., Mao, Y., Chow, L.C. and Proskin, H.M. (2000). "Fluoride in plaque fluid, plaque, and saliva measured for 2 hours after a sodium fluoride monofluorophosphate rinse." Caries Research **34**(5): 404-411.

Vogel, G.L., Tenuta, L.M.A., Schumacher, G.E. and Chow, L.C. (2010). "No calcium-fluoride-like deposits detected in plaque shortly after a sodium fluoride mouthrinse." Caries Research **44**(2): 108-115.

Vogel, G.L. (2011). Oral fluoride reservoirs and the prevention of dental caries. Fluoride and the Oral Environment. Buzalaf, M.A.R. **22:** 146-157.

Volgenant, C.M.C., van der Veen, M.H., de Soet, J.J. and ten Cate, J.M. (2013). "Effect of metalloporphyrins on red autofluorescence from oral bacteria." European Journal of Oral Sciences **121**(3 Pt 1): 156-161.

von der Fehr, F.R. (1967). "A study of carious lesions produced *in vivo* in unabraded, abraded, exposed, and F-treated human enamel surfaces, with emphasis on the x-ray dense outer layer." Archives of Oral Biology **12**(7): 797-814.

von der Fehr, F.R., Loe, H. and Theilade, E. (1970). "Experimental caries in man." Caries research **4**(2): 131-148.

Vratsanos, S.M. and Mandel, I.D. (1982). "Comparative plaque acidogenesis of caries-resistant vs caries-susceptible adults." Journal of Dental Research **61**(3): 465-468.

Wade, W. (2002). "Unculturable bacteria - the uncharacterized organisms that cause oral infections." Journal of the Royal Society of Medicine **95**(2): 81-83.

Wahl, M. (1989). "Marine epibiosis. 1. Fouling and antifouling - some basic aspects." Marine Ecology Progress Series **58**(1-2): 175-189.

Walden, D.C. and Wilensky, G.R. (1982). National health care expenditure study. Dental services: use, expenditures, and source of payment. Data preview 8. DHHS Publication. Department of Health and Human Services. Washington, D.C.**:** 82-3319.

Waller, E., de Josselin de Jong, E. and van der Veen, M. (2003). "QLF: A new diagnostic tool for oral health assessment." Woman Dentist Journal **1**(5): 8-16.

Walsh, T., Worthington, H.V., Glenny, A.-M., Appelbe, P., Marinho, V.C.C. and Shi, X. (2010). "Fluoride toothpastes of different concentrations for preventing dental caries in children and adolescents." Cochrane Database of Systematic Reviews(1).

Waltimo, T.M.T., Siren, E.K., Orstavik, D. and Haapasalo, M.P.P. (1999). "Susceptibility of oral *Candida* species to calcium hydroxide *in vitro*." International Endodontic Journal **32**(2): 94-98.

Waterhouse, J.C. and Russell, R.R.B. (2006). "Dispensable genes and foreign DNA in *Streptococcus mutans*." Microbiology **152**: 1777-1788.

Watson, G.K., Halliday, D., Albiston, I., Singleton, S. and Allison, C. (1995). An *in vitro* biofilm system for study of plaque ecology and physiology. The British Society for Dental Research (A Division of the IADR) 43rd Annual Meeting April 10-13, University of Manchester, Manchester, UK, Journal of Dental Research.

Watson, P.S., Pontefract, H.A., Devine, D.A., Shore, R.C., Nattress, B.R., Kirkham, J. and Robinson, C. (2005). "Penetration of fluoride into natural plaque biofilms." Journal of Dental Research **84**(5): 451-455.

Wegman, M.R., Eisenberg, A.D., Curzon, M.E.J. and Handelman, S.L. (1984). "Effects of fluoride, lithium, and strontium on intracellular polysaccharide accumulation in *S. mutans* and *A. viscosus*." Journal of Dental Research **63**(9): 1126-1129.

Weiss, S., King, W.J., Kestenbaum, R.C. and Donohue, J.J. (1965). "Influence of various factors on polysaccharide synthesis in *S. mitis*." Annals of the New York Academy of Sciences **131**(A2): 839-850.

Welin-Neilands, J. and Svensäter, G. (2007). "Acid tolerance of biofilm cells of *Streptococcus mutans*." Applied and Environmental Microbiology **73**(17): 5633-5638.

Welin, J., Wilkins, J.C., Beighton, D. and Svensater, G. (2004). "Protein expression by *Streptococcus mutans* during initial stage of biofilm formation." Applied and Environmental Microbiology **70**(6): 3736-3741.

West, N.X., Maxwell, A., Hughes, J.A., Parker, D.M., Newcombe, R.G. and Addy, M. (1998). "A method to measure clinical erosion: the effect of orange juice consumption on erosion of enamel." Journal of Dentistry **26**(4): 329-335.

Whitchurch, C.B., Tolker-Nielsen, T., Ragas, P.C. and Mattick, J.S. (2002). "Extracellular DNA required for bacterial biofilm formation." Science **295**(5559): 1487-1487.

White, D.J. (1987). "Reactivity of fluoride dentifrices with artificial caries. 1. Effects on early lesions - F-uptake, surface hardening and remineralisation." Caries Research **21**(2): 126-140.

White, D.J. (1992). "The comparative sensitivity of intraoral, *in vitro*, and animal-models in the profile evaluation of topical fluorides." Journal of Dental Research **71**: 884-894.

White, D.J., Cox, E.R. and Gwynn, A.V. (1995). "Effect of a stabilized stannous fluoride dentifrice on plaque acid (toxin) production." Journal of Clinical Dentistry **6**: 84-88.

Whitehead, S.A., Lo, L.Y., Watts, D.C. and Wilson, N.H.F. (1997). "Changes of surface texture of enamel *in vivo*." Journal of Oral Rehabilitation **24**(6): 449-453.

Whiteley, M., Lee, K.M. and Greenberg, E.P. (1999). "Identification of genes controlled by quorum sensing in *Pseudomonas aeruginosa*." Proceedings of the National Academy of Sciences of the United States of America **96**(24): 13904-13909.

Whitford, G.M., Wasdin, J.L., Schafer, T.E. and Adair, S.M. (2002). "Plaque fluoride concentrations are dependent on plaque calcium concentrations." Caries Research **36**(4): 256-265.

WHO (2012). "WHO Factsheet 318: Oral Health." **2014**.

Widstrom, E. and Eaton, K.A. (2004). "Oral healthcare systems in the extended European union." Oral Health & Preventive Dentistry **2**(3): 155-194.

Williams, J.L. (1897a). "A contribution to the study of pathology of enamel." Dental Cosmos **39**(4): 269-301.

Williams, J.L. (1897b). "A contribution to the study of pathology of enamel." Dental Cosmos **39**(3): 169-196.

Williams, J.L. (1897c). "A contribution to the study of pathology of enamel." Dental Cosmos **39**(5): 353-374.

Williams, R.A. (1964). "Biochemical aspect of adaptation to fluoride by micro-organisms isolated from dental plaque." Journal of Dental Research **43**(5SP): 946.

Wilson, M., Patel, H. and Noar, J.H. (1998). "Effect of chlorhexidine on multi-species biofilms." Current Microbiology **36**(1): 13-18.

Wimpenny, J., Manz, W. and Szewzyk, U. (2000). "Heterogeneity in biofilms." FEMS Microbiology Letters **24**(5): 661-671.

Wimpenny, J.W.T. and Colasanti, R. (1997). "A unifying hypothesis for the structure of microbial biofilms based on cellular automaton models." FEMS Microbiology Letters **22**(1): 1-16.

Winand, L., Dallemagne, M.J. and Duyckaerts, G. (1961). "Hydrogen bonding in apatitic calcium phosphates." Nature **190**(477): 164-165.

Winogradsky, S. (1928). "The direct method in soil microbiology and its application to the study of nitrogen fixation." Soil Science **25**: 37-43.

Wong, L. and Sissions, C.H. (2001). "A comparison of human dental plaque microcosm biofilms grown in an undefined medium and a chemically defined artificial saliva." Archives of Oral Biology **46**(6): 477-486.

Wood, J.M. (1967). "The amount, distribution and metabolism of soluble polysaccharides in human dental plaque." Archives of Oral Biology **12**(7): 849-858.

Wood, S.R., Kirkham, J., Marsh, P.D., Shore, R.C., Nattress, B. and Robinson, C. (2000). "Architecture of intact natural human plaque biofilms studied by confocal laser scanning microscopy." Journal of Dental Research **79**(1): 21-27.

Woodward, M. and Walker, A.R. (1994). "Sugar consumption and dental caries: evidence from 90 countries." British Dental Journal **176**(8): 297-302.

Woolley, L.H. and Rickles, N.H. (1971). "Inhibition of acidogenesis in human dental plaque *in situ* following use of topical sodium fluoride." Archives of Oral Biology **16**(10): 1187-1194.

Wright, D.E. and Jenkins, G.N. (1954). "The effect of fluoride on the acid production of saliva glucose mixtures." British Dental Journal **96**: 30-33.

Xie, H., Cook, G.S., Costerton, J.W., Bruce, G., Rose, T.M. and Lamont, R.J. (2000). "Intergeneric communication in dental plaque biofilms." Journal of Bacteriology **182**(24): 7067-7069.

Yamada, T., Hojo, S., Kobayashi, K., Asano, Y. and Araya, S. (1970). "Studies on the carbohydrate metabolism of cariogenic *Streptococcus mutans* strain PK-1." Archives of Oral Biology **15**(12): 1205-1217.

Yamada, T. and Carlsson, J. (1975). "Regulation of lactate dehydrogenase and change of fermentation products in streptococci." Journal of Bacteriology **124**(1): 55-61.

Yamada, T., Endo, K. and Araya, S. (1976). "A fructose 1,6-diphosphate-independent l-lactate dehydrogenase in a strain of *Streptococcus mutans*." Archives of Oral Biology **21**(4): 233-236.

Yamashita, Y., Kunimori, A. and Takehara, T. (1991). "Effect of calcium ions on cell-surface electrostatics of *Bacteroides gingivalis* and other oral bacteria." International Journal of Medical Microbiology Virology Parasitology and Infectious Diseases **275**(1): 46-53.

Yamashita, Y., Bowen, W.H., Burne, R.A. and Kuramitsu, H.K. (1993). "Role of the *Streptococcus mutans* gtf genes in caries induction in the specific-pathogen-free rat model." Infection and Immunity **61**(9): 3811-3817.

Yarwood, J.M. and Schlievert, P.M. (2003). "Quorum sensing in *Staphylococcus* infections." Journal of Clinical Investigation **112**(11): 1620-1625.

Yotis, W.W. and Brennan, P.C. (1983). "Binding of fluoride by oral bacteria." Caries Research **17**(5): 444-454.

Yotis, W.W., Zeb, M., McNulty, J., Kirchner, F., Reilly, C. and Glendenin, L. (1983). "Binding of F-18 by cell-membranes and cell-walls of *Streptococcus mutans*." Infection and Immunity **41**(1): 375-382.

Young, R.A. and Spooner, S. (1969). "Neutron diffraction studies of human tooth enamel." Archives of Oral Biology **15**: 47-63.

Zahradnik, R.T., Propas, D. and Moreno, E.C. (1978). "Effect of fluoride topical solutions on enamel demineralisation by lactate buffers and *Streptococcus mutans in vitro*." Journal of Dental Research **57**(9-10): 940-946.

Zameck, R.L. and Tinanoff, N. (1987). "Effects of NaF and SnF_2 on growth, acid and glucan production of several oral streptococci." Archives of Oral Biology **32**(11): 807-810.

Zarrinnia, K. and Kehoe, M.J. (1995). "The effect of different debonding techniques on the enamel surface - an *in vitro* qualitative study." American Journal of Orthodontics and Dentofacial Orthopedics **108**(3): 284-293.

Zaura, E., Buijs, M.J. and ten Cate, J.M. (2002). "The effects of the solubility of artificial fissures on plaque pH." Journal of Dental Research **81**(8): 567-571.

Zaura, E., Keijser, B.J.F., Huse, S.M. and Crielaard, W. (2009). "Defining the healthy "core microbiome" of oral microbial communities." BMC Microbiology **9**: 259-271.

Zaura, E., Buijs, M.J., Hoogenkamp, M.A., Ciric, L., Papetti, A., Signoretto, C., Stauder, M., Lingstrom, P., Pratten, J., Spratt, D.A. and Wilson, M. (2011). "The effects of fractions from shiitake mushroom on composition and cariogenicity of dental plaque microcosms in an *in vitro* caries model." Journal of Biomedicine and Biotechnology.

Zero, D.T., van Houte, J. and Russo, J. (1986a). "The intra-oral effect on enamel demineralization of extracellular matrix material synthesized from sucrose by *Streptococcus mutans*." Journal of Dental Research **65**(6): 918-923.

Zero, D.T., van Houte, J. and Russo, J. (1986b). "Enamel demineralization by acid produced from endogenous substrate in oral streptococci." Archives of Oral Biology **31**(4): 229-234.

Zero, D.T., Raubertas, R.F., Fu, J., Pedersen, A.M., Hayes, A.L. and Featherstone, J.D.B. (1992). "Fluoride concentrations in plaque, whole saliva, and ductal saliva after application of home-use topical fluorides." Journal of Dental Research **71**(11): 1768-1775.

Zero, D.T. (1995). "*In situ* caries models." Advances in Dental Research **9**(3): 214-230; discussion 231-214.

Zero, D.T. (2004). "Sugars - the arch criminal?" Caries Research **38**(3): 277-285.

Zero, D.T. (2006). "Dentifrices, mouthwashes, and remineralization/caries arrestment strategies." BMC Oral Health **6 Suppl 1**: S9-S9.

Zhang, J.Z., Harper, D.S., Vogel, G.L. and Schumacher, G. (2004). "Effect of an essential oil mouthrinse, with and without fluoride, on plaque metabolic acid production and pH after a sucrose challenge." Caries Research **38**(6): 537-541.

Zhang, X.Z., Anderson, P., Dowker, S.E.P. and Elliott, J.C. (2000). "Optical profilometric study of changes in surface roughness of enamel during *in vitro* demineralization." Caries Research **34**(2): 164-174.

ZoBell, C.E. (1936). "Periphytic habits of some marine bacteria." Proceedings of the Society for Experimental Biology and Medicine **35**(2): 270-273.

ZoBell, C.E. and Anderson, D.Q. (1936). "Observations on the multiplication of bacteria in different volumes of stored sea water and the influence of oxygen tension and solid surfaces." Biological Bulletin **71**(2): 324-342.

ZoBell, C.E., Anderson, D.Q. and Smith, W.W. (1937). "The bacteriostatic and bactericidal action of Great Salt Lake water." Journal of Bacteriology **33**(3): 253-262.

ZoBell, C.E. (1943). "The effect of solid surfaces upon bacterial activity." Journal of Bacteriology **46**(1): 39-56.